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Bifidobacterium bifidum Association with Changes in miR-196a/b-5p and Its Target Genes in HT-29 Colorectal Cancer Cells

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ABSTRACT

Background and Aim: Probiotics, particularly species of Bifidobacterium, have shown potential anticancer properties through mechanisms involving immune modulation, regulation of apoptosis, and production of bioactive metabolites. This study aimed to examine the cytotoxic effects of post-fermentation medium (PFM) and cell extract (CE) derived from *Bifidobacterium (B.) bifidum* on colorectal cancer (CRC) cells and to evaluate their impact on the expression of cancer-related microRNAs (miRNAs) and target genes.

Materials and Methods: HT-29 colorectal cancer cells were exposed to different concentrations (0–50% v/v) of PFM and CE. Cytotoxicity was assessed using the MTT assay. The expression levels of miR-196a-5p and miR-196b-5p and their target genes (*HOXB8*, *IGF2BP3*, and *E2F7*) were quantified using RT-qPCR.

Results: PFM induced a dose-dependent reduction in HT-29 cell viability, with an IC_{50} of approximately 35%, while CE displayed minimal cytotoxicity. PFM also upregulated miR-196a-5p and miR-196b-5p, particularly at 35% and 50%, whereas CE had no significant effect. Correspondingly, PFM was associated with significant downregulation expression of *HOXB8* and *IGF2BP3*. No meaningful changes were observed in *E2F7* expression or in CE-treated cells.

Conclusion: *Bifidobacterium bifidum*-derived PFM demonstrated anticancer activity by reducing cancer cell viability and showing an association with changes in miRNA and target gene expression. However, these findings are correlative and do not establish a causal, miRNA-mediated mechanism. Further functional studies (e.g., using miRNA mimics/inhibitors or gene knockdown) are required to determine whether the observed gene expression changes are directly mediated by miRNA modulation, and *in vivo* validation is also warranted.

Keywords: *Bifidobacterium bifidum*, Colorectal Neoplasms, Fermentation Products, Gene Expression Regulation, MicroRNAs

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1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality worldwide. This malignancy arises from a complex interaction between genetic susceptibility, environmental influences, and disturbances in the gut microbiota **(1)**. Recent developments in cancer research have emphasized the crucial importance of the intestinal microbiome in modulating physiological processes relevant to tumor development. Among the various microbial inhabitants *Bifidobacterium (B.) bifidum*, a prominent probiotic species in the human gut, has gained increasing attention for its potential involvement in cancer-related mechanisms. *B. bifidum,* widely

recognized for its beneficial effects on gastrointestinal health, exhibits several properties that may contribute to its anticancer activity, including strengthening the intestinal barrier, modulating host immune responses, and maintaining microbial homeostasis **(2-4)**.

Notably, emerging evidence suggests that the influence of probiotics extends to the regulation of gene expression through epigenetic mechanisms, particularly via the modulation of microRNAs (miRNAs) **(5)**.

The miRNAs are tiny non-coding RNAs that influence the regulation of genes expression at post-transcriptional level. This regulation plays critical roles in various health and disease states .Among them, miR-196a/b-5p has attracted noteworthy consideration due to its oncogenic role in several types of cancer. Increased levels of miR-196a/b-5p have been related to enhanced tumor cell growth, invasion, and resistance to programmed cell death **(6, 7)**. In CRC, aberrant levels of miR-196a/b-5p contribute to the maintenance of an oncogenic phenotype **(8, 9)**. Thus, targeting dysregulated miRNA expression may provide new therapeutic strategies for the colorectal cancer.

Several target genes of miR-196a/b-5p have been implicated in tumorigenesis. *HOXB8*, a homeobox transcription factor, is overexpressed in CRC and enhances proliferation of cells and their invasive behavior metastasis, and chemotherapy resistance via the Wnt/β-catenin and STAT3 signaling pathways. Insuline-like growth factor 2 mRNA-binding protein 3)IGF2BP3) stabilizes oncogenic mRNAs by recognizing their N^6-methyladenosine (m^6A) modifications and is related to tumor growth, metastasis, and unfavorable outcomes across multiple cancers **(10)**. The *E2F7* gene, a transcriptional regulator of the E2F family, is frequently upregulated in CRC, where it modulates cell cycle progression and may influence proliferation, although its precise role remains context-dependent **(11)**. Understanding the modulation of these genes may clarify the mechanisms by which probiotics influence cancer biology.

This study investigates the regulatory effects of *B. bifidum* on miR-196a/b-5p in HT-29 CRC cells. It also examines how these miRNAs influence the level of important target genes expression that are involved in tumor progression.

The HT-29 cell line is a well-established *ex vivo* model for studying colorectal tumor biology due to its tumorigenic characteristics **(12, 13)**. Despite increasing interest in probiotic–cancer interactions, the molecular mechanisms by which *B. bifidum* modulates miRNAs—particularly miR-196a/b-5p and its target genes remain largely unexplored. This study represents the first inquiry into the impact of *B. bifidum* on the level expression of miR-196 a/b-5p and its key target genes—*HOXB8, E2F7,* and *IGF2BP3*—which are critically involved in CRC cell proliferation, invasion, and cell death. Considering the essential role of miRNAs in cancer biology and the growing interest in microbiota-based therapeutics, elucidating how *B. bifidum* modulates miR-196a/b-5p and its downstream targets could pave the way for novel miRNA-targeted therapies or improve existing CRC treatments.

2. Materials and Methods

2.1 Bacterial Strain and Culture Conditions

Bifidobacterium (B.) bifidum (strain 1644 PTCC) was obtained in a pure, lyophilized form from the Iranian Industrial Microbial Culture Collection (Iran Scientific and Industrial Research Organization). The bacterium was revived and grown in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany). The cultures were incubated anaerobically at 37°C for 72 hr using an anaerobic jar and gas pack system (AnaeroGen™, Oxoid, UK).

2.2 Preparation of Post-Fermentation Medium (PFM)

To prepare the post-fermentation medium (PFM), an active culture of *B. bifidum* was inoculated into fresh MRS broth and incubated for 24 hr at 37°C under anaerobic conditions. The culture was centrifuged at 10,000×g for 15 min at 4°C. In the next step, the cell-free supernatant was obtained. The pH level was calibrated to 7.0 ± 0.1 using 1N NaOH or HCl, measured with a pH meter (Metrohm 827 pH Lab, Switzerland). Filtration through a 0.22 µm syringe filter (Sartorius, Germany) was employed to sterilize the supernatant under a Class II laminar flow cabinet. The filtered PFM was kept in sterile, capped tubes at −20°C until further use. For the cell treatment experiments, HT-29 cells treated with fresh MRS medium (without bacterial culture) were included as negative control to account for any effect of the medium alone **(14, 15)**.

2.3 Preparation of Cell Extract (CE)

To prepare the bacterial cell extract (CE), *B. bifidum* was cultured as described above. After incubation, the cells were collected by centrifugation at 10,733×g for 15 min at 4°C. The cell pellets were washed three times with sterile phosphate-buffered saline (PBS) and centrifuged again. The washed cells were lysed using an ultrasonic bath sonicator (5 min, 50% amplitude; pulse on: 6 s; off: 2 s) keeping in ice bath. Further

homogenization was performed using a manual homogenizer to ensure complete disruption. The lysate was adjusted to pH 7.1 ± 0.1 and sterilized through 0.22 µm filtration. The final CE was stored at −20°C until utilization **(15)**.

2.4 Cell Culture and Treatment

The HT-29 human colorectal adenocarcinoma cell line (NCBI code: C466) was obtained from the Pasteur Institute of Iran. Cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S), under standard conditions (37°C in a humidified incubator with 5% CO_2).

For the treatment, cells were seeded into 6-well plates (5×10^5 viable cells/well) (counted using the trypan blue exclusion method). Following 24-hr incubation to reach semi-confluency, the medium was replaced with fresh RPMI-1640 containing 10–50% (v/v) PFM or CE. Each treatment condition was performed in three independent biological replicates (n = 3), conducted on different days with freshly seeded HT-29 cells.

Within each biological replicate, MTT measurement was performed in technical triplicates, and qPCR assay was conducted in technical duplicates to ensure the accuracy and reproducibility **(16)**. Cells were incubated for 48 hr under standard conditions before harvesting, and media were removed prior to further analysis. Data are presented as the mean ± SD of the three biological replicates **(15)**.

2.5 MTT Cell Viability Assay

To assess the cytotoxic effects of PFM and CE, MTT assay was carried out wherein cells were placed 96-well plate (10,000 cell/well) and allowed to incubate overnight. On the subsequent day, the cells received treatments with PFM or CE at concentrations between 10% and 50% (v/v) for 48 hr. Then, 20 µL of MTT solution (5 mg/mL in PBS; Sigma-Aldrich, Cat# M5655) was added to each well and incubated for 4 hr at 37°C. Following incubation, the medium was gently aspirated, and 200 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the formazan crystals. The optical density (OD) was then measured at 570 nm, with background absorbance at 630 nm subtracted. The percentage of cell viability was assessed with the following formula: Viability (%) = (Abs_sample / Abs_control) × 100.

2.6 RNA Extraction and Real-Time qPCR

After 48 hr of treatment, total RNA was extracted using the Pars Tous RNA Extraction Kit (Cat# A101231, Iran) according to the manufacturer's instructions. The concentration and purity of the extracted RNA were evaluated spectrophotometrically.

Complementary DNA (cDNA) synthesis for mRNA targets was performed using the Ad Bio cDNA Synthesis Kit (Cat# RT-20, Korea). For miRNA analysis, cDNA was synthesized using the All-in-One miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, Cat# QP015, Canada), which includes proprietary miRNA-specific primers and a universal reverse primer. It was used strictly according to the manufacturer's protocol.

Quantitative real-time PCR (RT-qPCR) was carried out using SYBR Green Master Mix (Yekta Tajhiz Azma, Cat# YT2552, Iran) on a Rotor-Gene Q real-time PCR system (Qiagen, Germany). Primer pairs for all mRNA targets were custom-designed based on reference mRNA sequences obtained from the NCBI database. All mRNA primers were validated in our laboratory by the standard and melt curve analyses to confirm amplification efficiency (90–105%) and specificity.

GAPDH and RNU6 were used as internal reference genes for normalization of mRNA and miRNA expression levels, respectively. All qPCR reactions were performed in technical duplicates to ensure reproducibility and measurement accuracy. Relative gene expression levels were calculated using the 2^−ΔΔCT method (Table 1).

2.7 Statistical Analysis

All data are presented as mean ± standard deviation (SD) from three biological replicates (n = 3 independent experiments). Technical replicates (triplicates for MTT and duplicates for qPCR) were performed within each biological replicate to ensure the measurement precision. Statistical analyses were performed using GraphPad Prism 9.0. Group comparisons were evaluated by one-way ANOVA followed by Tukey's *post-hoc* test. A P-value<0.05 was considered statistically significant. The Shapiro–Wilk test was used to assess data normality.

Table 1. Primer sequences.

Gene symbol	NCBI number	Forward primer (5'–3')	Reverse Primer (5'–3')
GAPDH	NM_0020467	CTCAGACACCATGGGGAAGGTGA	ATGATCTTGAGGCTGTTGTCATA
IGF2BP3	NM_006547	TCGTGACCAGACACCTGATGAG	GGTGCTGCTTTACCTGAGTCAG
E2F7	NM_203394	TCTGAACCCGACTGTCCCTCTT	TTTGGCAGCCACATCCAGAGTG
HOXB8	NM_024016	GTCGCCCACACAGCTCTTCCC	AATAGGAACTCCTTCTCCAGCTC
RNU6-F	NR_0030272	Proprietary (GeneCopoeia kit)	Universal primer (kit)
miR-196a-5p	MI:0000226	Proprietary (GeneCopoeia kit)	Universal primer (kit)
miR-196b-5p	MI:0001080	Proprietary (GeneCopoeia kit)	Universal primer (kit)

3. Results

3.1 Cytotoxic Effects of PFM and CE on HT-29 Cells

To assess the cytotoxic potential of post-fermentation medium (PFM) and cell extract (CE; sterile lysate of non-fermented *B. bifidum*), HT-29 cells were exposed to increasing concentrations (0–50% v/v) of each treatment for 48 hr, followed by MTT assay. HT-29 cells treated with fresh MRS medium alone were considered as negative control.

As shown in Figure 1 and Table 2, PFM reduced HT-29 cell viability in a concentration-dependent manner, with the most notable decline at 40% and 50% (41% ± 4.2 and 33% ± 5.5, respectively; $P<0.01$). The calculated IC_{50} was approximately 35% v/v, indicating a moderate cytotoxic threshold. In contrast, CE did not significantly affect cell viability at any tested concentration (all values >89%; $P>0.05$), suggesting that non-fermented bacterial lysates lack the cytotoxic components present in PFM.

3.2 Impact of PFM and CE on miR-196a/b-5p Expression in HT-29 Cells

To evaluate whether *B. bifidum*-derived fractions are associated with changes in miR-196a-5p and miR-196b-5p expression, HT-29 cells were exposed to different concentrations (0%, 20%, 35%, and 50% v/v) of PFM or CE for 48 hr. The expression levels were measured via RT-qPCR and normalized to RNU6. Cells treated with fresh MRS medium alone served as negative control.

As summarized in Figure 2 and Table 3, PFM treatment at 35% concentration increased the expression of miR-196a-5p (1.98 ± 0.12) and miR-196b-5p (1.58 ± 0.12) significantly compared to control ($P<0.05$). A slight decrease was observed at 50%, but expression levels remained higher than in untreated cells. CE treatment did not significantly affect miRNA expression at any tested concentration ($P>0.05$).

3.3 Expression of miR-196a/b-5p Target Genes Following PFM and CE Treatment

To investigate the molecular mechanisms associated with observed changes in miRNA, the mRNA expression levels of three established miR-196a/b-5p targets—*HOXB8*, *IGF2BP3*, and *E2F7*—were measured in HT-29 cells exposed to various concentrations of PFM and CE for 48 hr. The expression levels were normalized to GAPDH and compared with untreated controls.

PFM treatment resulted in a significant downregulation of *HOXB8* and *IGF2BP3*, particularly at a 35% concentration, whereas *E2F7* expression remained relatively unchanged under all conditions. CE treatment did not significantly affect the expression of any target genes at any tested concentration (Figure 3 and Table 4).

Table 2. The impact of CE and PFM concentrations on HT-29 cells viability.

Concentration (v/v)	PFM (%)	CE (%)
0	100	100
10	90±2	98±1.3
20	85±5	95±2
30	65±3*	96±4
40	41±4.2**	90±5
50	33±5.5**	89±2.5

Statistical significance: P<0.05 (*), P<0.01 (**)

Table 3. Effect of CE and PFM on expression level of miR-196a-5p and miR-196b-5p in HT-29 cells (fold change ± SD).

Concentration (v/v)	Fold change of PFM		Fold change of CE	
	miR-196b-5p	miR-196a-5p	miR-196b-5p	miR-196a-5p
0	1	1	1	1
20	1.23±0.23	1.03±0.23	1.03±0.13	1.08±0.13
35	1.98±0.12*	1.58±0.12*	1.08±0.02	1.28±0.02
50	1.79±0.31	1.6±0.31	1.19±0.21	1.25±0.21

Statistical significance: P<0.05 (*)

Table 4. Fold change in expression levels of *IGF2BP3, E2F7*, and *HOXB8* genes in HT-29 cells treated with CE and PFM at different concentrations (v/v).

Concentration (v/v)	Fold change of PFM			Fold change of CE		
	HOXB8	*E2F7*	*IGF2BP3*	*HOXB8*	*E2F7*	*IGF2BP3*
0	1	1	1	1	1	1.07
20	0.9	0.95	1.08	1.18	1.04	1.10
35	0.46**	1.11	0.77*	1.12	0.89	1.05
50	0.42	0.97	0.80	1.06	0.93	1.21

Statistical significance: P<0.05 (*), P<0.01 (**)

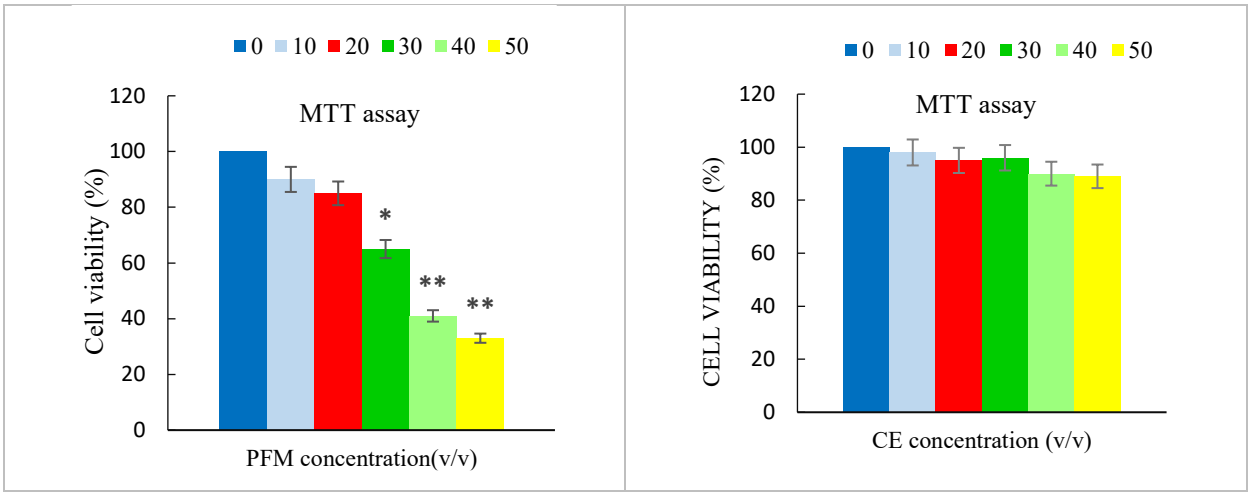


Figure 1. Effects of PFM and CE on HT-29 cell viability measured by MTT assay. Data are presented as mean ± SD from three independent biological replicates (n = 3). (A) PFM treatment caused a dose-dependent decrease in cell viability (IC_{50} ≈ 35% v/v). (B) CE showed no significant effect on viability (*P*>0.05 vs. control). Statistical significance: P<0.05 compared to untreated control **(Prepared by Authors, 2026)**.

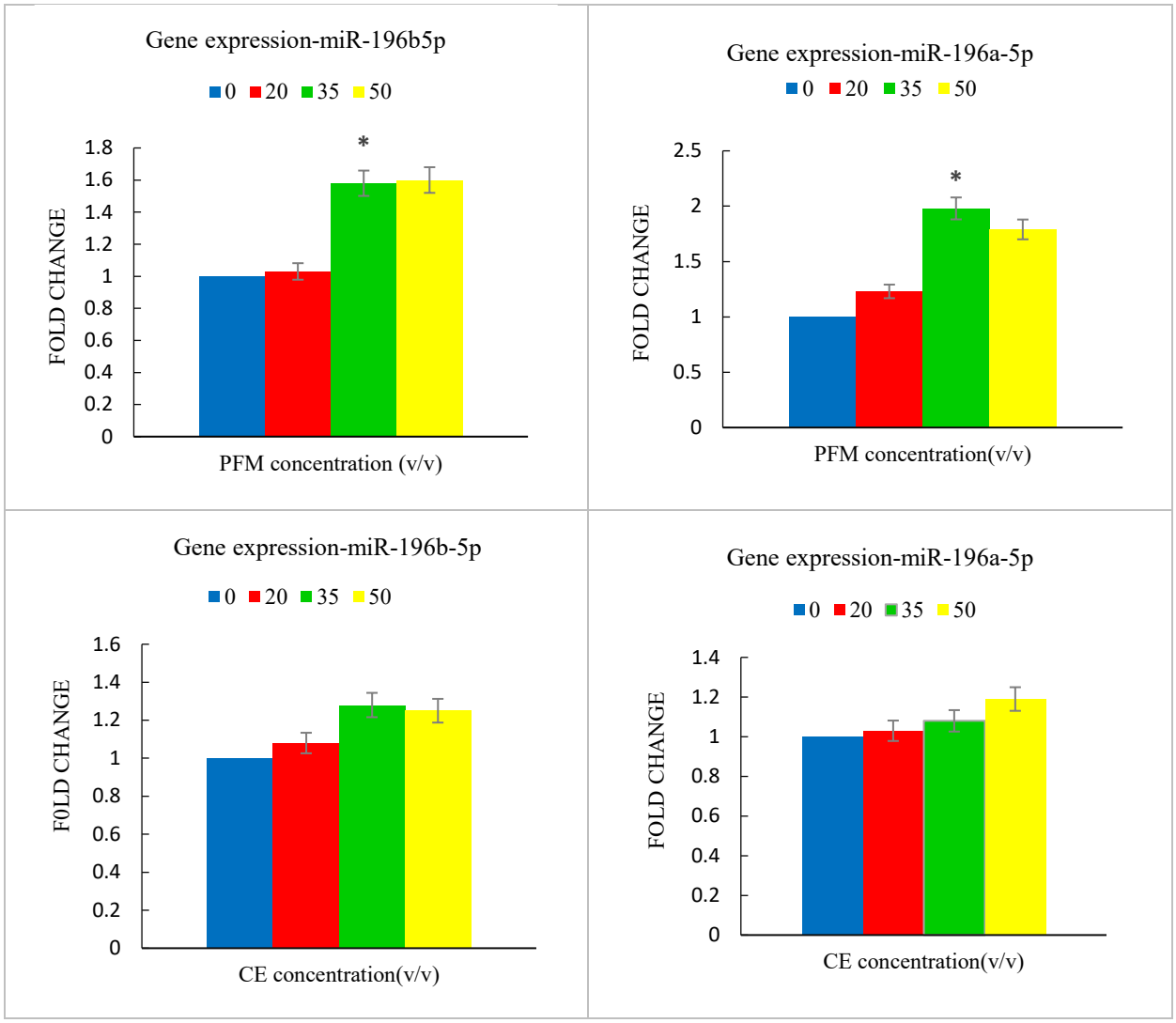
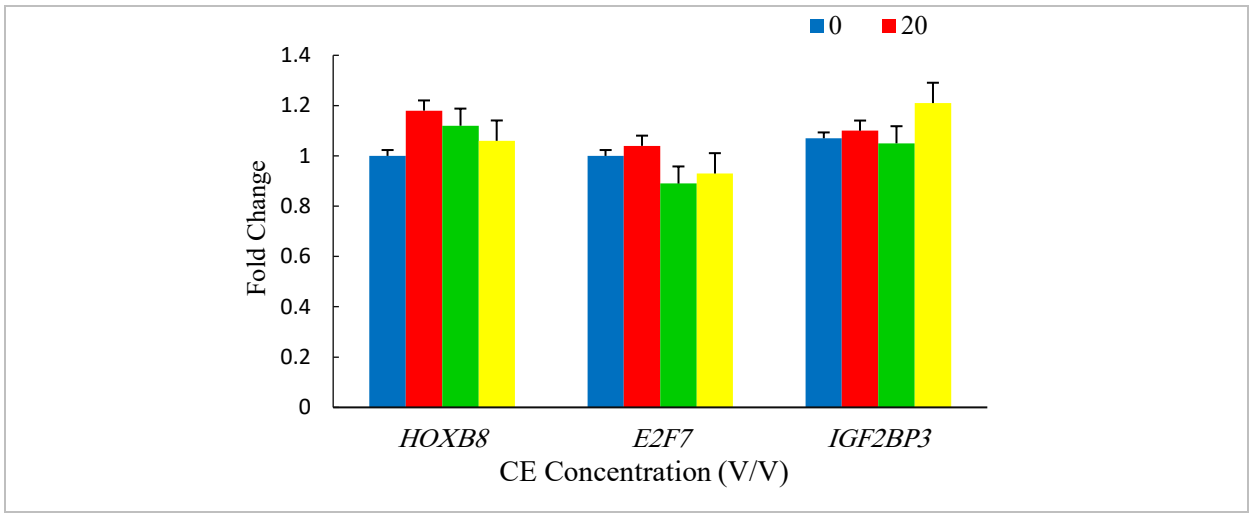


Figure 2. Effects of PFM and CE on miR-196a-5p and miR-196b-5p expression in HT-29 cells. Cells were treated with 0%, 20%, 35%, and 50% (v/v) for 48 hr. Expression levels were measured by RT-qPCR and normalized to RNU6. Data are presented as fold change ± SD from three independent biological replicates (n = 3). (A, B) PFM treatment was associated with increased miRNA expression at 35% (P<0.05). (C, D) CE treatment showed no significant effect (P>0.05) **(Prepared by Authors, 2026)**.



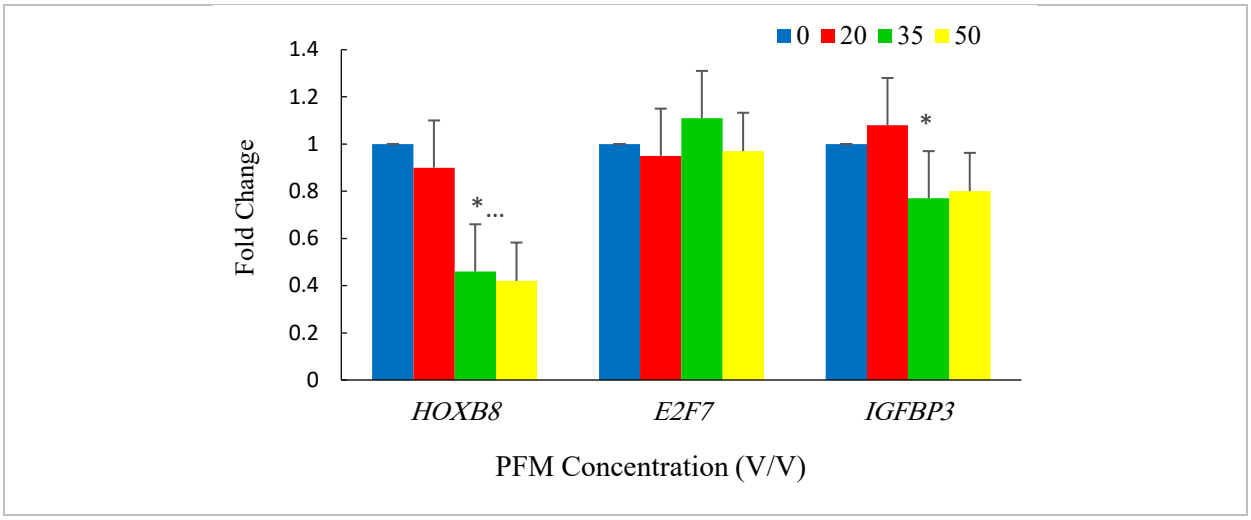


Figure 3. Effects of PFM (A) and CE (B) on the expression of *HOXB8*, *IGF2BP3*, and *E2F7* in HT-29 cells. Gene expression was quantified by RT-qPCR and normalized to GAPDH. Data are presented as fold change ± SD from three independent biological replicates (n = 3), relative to untreated control (0%). **(A)** PFM treatment decreased *HOXB8* and *IGF2BP3* expression at 35% and 50%, while *E2F7* expression remained unchanged. **(B)** CE treatment did not significantly alter gene expression at any concentration (P>0.05) **(Prepared by Authors, 2026)**.

4. Discussion

While probiotics have been reported to exert anticancer effects through various mechanisms, the present study provides correlative observations between *B. bifidum*-derived PFM treatment and changes in miRNA and target gene expression. The precise mechanistic pathway remains hypothetical and requires further functional validation.

In recent years, probiotic microorganisms, particularly Lactobacillus and Bifidobacterium species have gained considerable attention for their anticancer potential. Their effects are mediated through multiple mechanisms, including the production of antioxidant enzymes, scavenging reactive oxygen species (ROS), heavy metal chelation, and neutralization of carcinogens **(17, 18)**. Additionally, probiotics can regulate cell cycle in cancer cells, suppress proliferation, and enhance sensitivity to apoptosis, all of which are crucial processes in tumor suppression **(19)**.

Colorectal cancer (CRC) is characterized by uncontrolled cell proliferation and evasion of apoptosis, leading to tumor progression. Emerging evidence highlights the capacity of Bifidobacterium species to modulate genes involved in apoptotic and anti-apoptotic pathways, suggesting potential therapeutic opportunities **(20)**.

In this study, the post-fermentation medium (PFM) derived from *B. bifidum* exhibited notable cytotoxic effects on HT-29 colorectal cancer cells in a dose-dependent manner with an IC_{50} of approximately 35% (v/v), whereas the bacterial cell extract (CE) showed minimal cytotoxicity. Gene expression and cytotoxic responses varied across concentrations, likely due to non-linear dose-response effects and differential sensitivities of target genes.

Secreted metabolites present in PFM, such as short-chain fatty acids (SCFAs), proteins, and bacteriocins may play key roles in mediating the observed anticancer activity **(21)**.

Mechanistically, butyrate acts as a histone deacetylase (HDAC) inhibitor, altering chromatin structure and enhancing transcription of pro-apoptotic genes, including BAX and p21, thereby promoting apoptosis and inhibiting cell proliferation **(22)**.

PFM treatment was associated with a moderate but significant upregulation of miR-196a/b-5p. Despite their sequence similarity, miR-196a-5p and miR-196b-5p differ in tissue-specific expression and biological function **(6, 23)**.

Previous studies have reported upregulation of miR-196a in head and neck **(23)**, oral **(24)**, gastric **(25)**, pancreatic **(6, 26)**, cervical **(27)**, and lung **(8)** cancers, while downregulation of miR-196b in oral **(23)**, gastric **(28)**, colorectal **(29)**, glioblastoma, and leukemia **(23)** cancers. Both miRNAs are implicated in oncogenic processes, enhancing proliferation, migration, invasion, and resistance to therapy, though tumor-suppressive functions have also been reported in certain contexts, such as melanoma and breast cancer **(30)**. Similarly, chemotherapeutic agents like Epirubicin can induce specific miRNAs, including miRNA-22 and miRNA-331, affecting cell survival and metastasis in triple-negative breast cancer **(31)**.

To evaluate downstream effects, the expression levels of established miR-196 target genes—*HOXB8*, *IGF2BP3*, and *E2F7*—were measured. PFM treatment led to significant downregulation of *HOXB8* and *IGF2BP3*, while *E2F7* remained unchanged. Although these results suggest a potential link between PFM treatment and miR-196a/b-5p–associated regulation of *HOXB8* and *IGF2BP3*, the observations are correlative, and direct causality has not been experimentally confirmed.

The extent of target gene suppression may also be influenced by other bioactive metabolites in PFM, such as SCFAs, and is not proven to be solely mediated by miRNA changes **(32)**. Functional studies using miRNA mimics, inhibitors, or gene knockdown are required to validate these regulatory relationships.

HOXB8 contributes to CRC progression by promoting proliferation and migration via the Wnt/β-catenin pathway **(33)**. Elevated *HOXB8* correlates with poor prognosis and is observed even in precancerous lesions **(34)**. It promotes epithelial–mesenchymal transition (EMT) and activates STAT3 signaling, enhancing oncogenic drivers like c-Myc and Cyclin D1 **(35, 36)**. Importantly, the ratio of miR-196 to *HOXB8* can modulate sensitivity to FOLFOX4 chemotherapy in CRC, where increased miR-196 levels correlate with reduced *HOXB8* and improved chemotherapeutic response **(37)**.

IGF2BP3, an oncofetal RNA-binding protein, is associated with tumor progression and poor prognosis in multiple cancers **(38, 39)**. It stabilizes oncogenic mRNAs, including RCC2 and *EGFR*, promoting survival, chemoresistance, and proliferation **(40, 41)**. Bioinformatic analyses identify *IGF2BP3* as a target of miR-196b-5p **(42, 43)**.

This study provides the first evidence that *B. bifidum*-derived PFM downregulates *IGF2BP3* in colorectal cancer cells, potentially via miR-196a/b-5p, though functional validation is necessary.

Conversely, CE treatment did not significantly affect miRNA or target gene expression, reinforcing that secreted metabolites, rather than bacterial cellular components, are likely responsible for the observed anticancer activity. Notably, at 50% PFM, miRNA upregulation and *IGF2BP3* downregulation were slightly less pronounced than at 35%, despite greater cytotoxicity. This non-linear response may result from impaired cellular machinery required for miRNA biogenesis and mRNA regulation under highly cytotoxic conditions.

The present study has several limitations. First, the observed changes in miR-196a/b-5p and their target genes are correlative and do not establish a direct mechanistic link. Second, experiments were performed using a single colorectal cancer cell line (HT-29), which may limit the generalizability of the findings. Future studies should include functional validation using miRNA mimics, inhibitors, or gene knockdown, as well as testing additional CRC to confirm the selectivity and reproducibility of the observed effects.

The other limitation of the study is the lack of evaluation of PFM and CE effects on normal colorectal epithelial cells. Functional validation of the proposed miRNA-mediated regulatory mechanism was not performed. Future investigations should include non-cancerous colon epithelial cell lines to assess selective toxicity and further clarify safety and therapeutic potential.

5. Conclusion

This study demonstrates that the post-fermentation medium (PFM) derived from *Bifidobacterium bifidum* exerts significant anticancer effects on HT-29 colorectal cancer cells. PFM treatment reduced cell viability in a dose-dependent manner and was associated with upregulation of miR-196a/b-5p and downregulation of their known oncogenic targets, *HOXB8* and *IGF2BP3*. In contrast, the bacterial cell extract (CE) showed negligible effects on these parameters. These findings indicate that fermentation-derived bioactive metabolites may contribute to the observed anticancer activity. However, the observed relationships between PFM, miRNA changes, and target gene expression are correlative and do not establish a direct mechanistic link. The substantial gene downregulation observed may also be influenced by other bioactive compounds in PFM (e.g., SCFAs with HDAC-inhibitory activity), independent of -or only partially dependent on- the modest miRNA changes. Therefore, these mechanistic interpretations remain hypothesis-generating. Future functional studies using miRNA mimics, inhibitors, or gene knockdown are required to validate whether miR-196a/b-5p directly mediates the observed gene expression changes, and *in vivo* investigations are needed before drawing therapeutic conclusions.

6. Declarations

6.1 Acknowledgment

The authors gratefully acknowledge Dr. Shekufe Rezghi Barez (Isfahan University of Medical Sciences, Iran) for her assistance in experimental procedures.

6.2 Ethical Considerations

Not applicable. This study did not involve human participants or animal experiments.

6.3 Authors' Contributions

Somayeh Farahmand: Conceptualization, methodology, software, validation, investigation, data curation, writing — original draft, review & editing, visualization, supervision. Arghavan Sabzevari: Conceptualization, methodology, software, formal analysis, investigation, resources, data curation, writing — original draft, review & editing, visualization, supervision, project administration, funding acquisition. Mohammad Amin Nicknezhad: Conceptualization, review & editing, supervision.

6.4 Conflict of Interests

The authors report there are no competing interests to declare.

6.5 Financial Support and Sponsorship

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

6.6 Using Artificial Intelligence Tools (AI Tools)

The authors used AI-based language tools (e.g., grammar or style checkers) to improve the readability of the manuscript. All content, interpretations, and conclusions are solely the work of the authors.

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