

ENHANCED APOPTOSIS VIA AKT/P53 AND CELL CYCLE ARREST VIA CYCLIN MODULATION IN HT-29 CELLS BY *BACTEROIDES FRAGILIS* SUPERNATANT: A COMPARATIVE STUDY WITH LYSATE

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ABSTRACT

Bacteroides fragilis, a common member of the human gut microbiome, has recently get attention for its potential anti-cancer effects and its promise as a next-generation probiotic. In this study, we evaluated the impact of *B. fragilis* supernatant and lysate on human colorectal cancer HT-29 cells. Cytotoxic effects were measured using the MTT assay, while changes in gene expression (including cyclins, apoptotic markers, and AKT) were analyzed using real-time PCR. Additionally, flow cytometry was used to assess apoptosis and alterations in cell cycle progression. Results showed a time-dependent reduction in cell viability, with the supernatant consistently showing a stronger effect than the lysate. Flow cytometry analysis revealed increased apoptosis and significant accumulation of cells in the sub-G1 phase, particularly after 48 hours of treatment. Gene expression analysis further confirmed upregulation of pro-apoptotic markers (*Caspase-9*, *Caspase-2*, *p53*, and *p21*) and downregulation of *cyclins* and the anti-apoptotic gene *AKT*. These findings suggest that *B. fragilis* extracts—especially the supernatant—can induce apoptosis and disrupt cell cycle progression in colorectal cancer cells. This highlights the potential of utilizing bacterial secretomes as the therapeutic potential in cancer treatment.

Keywords: *Bacteroides fragilis*, colon cancer, cell cycle, apoptosis, gene expression

INTRODUCTION

Probiotics offer a range of health benefits, including immune regulation, antioxidant effects, and the ability to suppress harmful pathogens by competing for nutrients, adhering to the intestinal lining, and producing antimicrobial substances like bacteriocins. Both in vitro and in vivo studies have highlighted their anticancer and anti-inflammatory properties, as well as their role in preserving mucosal integrity. These functions are closely connected to colorectal cancer (CRC), supporting the potential of probiotics as complementary therapy (Bhatia A, 2025; Sarita, Samadhan, Hassan, & Kovaleva, 2025; Stavrou & Kotzampassi, 2025). Bacteroidota, a dominant phylum in the gut microbiota, includes key genera such as *Bacteroides*, *Parabacteroides*, *Prevotella*, and *Alistipes*. Although these microbes play a central role in maintaining intestinal and overall health, some strains are opportunistic pathogens and may pose risks, particularly in compromised individuals. Dysbiosis involving Bacteroidota has been associated with various disorders, including IBD and metabolic syndromes. Despite these concerns, their therapeutic potential is gaining attention, with regulatory bodies like the European Commission currently evaluating their safety for use in food products (Aziz, Nageen, Zunaira, & Lin, 2024). Also, the ongoing exploration of Bacteroidota underscores their promise as both biomarkers and active agents in future microbiome-based therapies. In particular, *Bacteroides* species have emerged as promising next-generation probiotics due to their strong interactions with the host immune modulation and their influence on metabolic pathways and gut homeostasis within the gut microbiota (Muhammad Aammar Tufail & Ruth A. Schmitz, 2025). They are essential for digesting complex carbohydrates and producing short-chain fatty acids (SCFAs) like butyrate—compounds that support gut barrier function, fuel colonocytes, and help regulate inflammation (Cong, Zhou, & Zhang, 2022). *Bacteroides fragilis* BF839, a probiotic strain used clinically in China, has shown potential to enhance cancer immunotherapy. In a study, BF839 improved sensitivity to immune checkpoint inhibitors (ICIs) by enriching the gut microbiota and promoting CD8⁺ T cell infiltration. RNA-seq analysis revealed activation of the cGAS–STING pathway, which was confirmed to mediate BF839's antitumor effects. BF839 also worked synergistically with anti-PD-1 therapy, leading to significant tumor regression. These findings highlight BF839 as a promising adjunct to boost ICI efficacy in solid tumors (Peng et al., 2025). The cell cycle is tightly regulated by a coordinated network of proteins, with cyclins and their catalytic partners, cyclin-dependent kinases (CDKs), at its core. Cyclin levels rise and fall at specific stages, controlled by transcriptional activation and, ensuring orderly entry into and progression through each phase of the cell

cycle. Each phase of the cell cycle is governed by unique cyclin-CDK pairs: Cyclin D-CDK4/6: Active in early G1, integrating external mitogenic signals to initiate cell cycle entry. Cyclin E-CDK2: Drives the G1/S transition, and allow DNA replication. Cyclin A-CDK2: Functions through S phase to promote DNA synthesis. Cyclin A/B-CDK1: Controls the initiation and orderly progression of mitosis. Synergistic effects have been observed when Cyclin-CDK inhibitors are paired with agents targeting the PI3K/AKT pathways as well as with Caspase 9 + P21 and P53 apoptotic components (Glaviano et al., 2025; Lim, Dorstyn, & Kumar, 2021; Pellarin et al., 2025).

The present study aimed to investigate how the supernatant and lysate of *Bacteroides fragilis* affect human colorectal cancer HT-29 cells. Our results show that exposure to *B. fragilis* triggers the activation of genes involved in promoting apoptosis, while suppressing cyclin gene expression, which disrupts normal cell cycle progression and promotes apoptosis. Together, these effects point to the potential of *B. fragilis* to help inhibit the development of colorectal cancer.

Material and Methods

Bacterial cultivation, sonicate and supernatant extraction, and HT-29 cell line culture

Bacteroides fragilis (ATCC 23745) was obtained from the Pasteur Institute of Iran in freeze-dried form and cultured under anaerobic conditions. The bacterial pellet was rehydrated with brain heart infusion (BHI) broth, incubated briefly at 37°C, and then transferred to fresh BHI for growth. After confirming purity on BHI agar, the culture was incubated anaerobically for 16 hours. Bacterial growth was monitored by measuring optical density at 600 nm, and cells were harvested during the exponential phase. The supernatant was collected by centrifugation at 1700 rpm for 15 min and sterile filtration for further use. Bacterial pellet was washed in PBS (Sigma-Aldrich). 1-ml of *B. fragilis* (at a concentration of 10⁸ cfu/ml) lysed on ice by sonication using a Hielscher Sonicator. The lysate filtered through a 0.22-µm filter membrane (Biofil).

The human colorectal cancer cell line HT-29 was obtained from the National Cell Bank of Iran. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (v/v). Cultures were maintained at 37°C in a humidified incubator set to 5% CO₂ and 95% air.

MTT Assay Analysis of HT-29 Cells Treated with *Bacteroides fragilis* Supernatant and Lysate (*B.f Su&Ly*)

The cytotoxic effect of *B. fragilis* supernatant and lysate on HT-29 cells was evaluated using the MTT assay. Cells (5×10^4) were seeded in 96-well plates and incubated overnight. They were then treated with 10% (10^8 CFU/mL) of *B. fragilis* supernatant and lysate, while control wells received only RPMI 1640 medium. After 24 and 48 hours, MTT solution (0.5 mg/mL) was added, and cells were incubated for 1 hour at 37 °C. Formazan crystals were dissolved in DMSO and absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated relative to controls.

RNA extraction and Gene Expression Analysis

It was followed the MIQE guidelines (Bustin et al., 2009) in the real time processes. To assess gene expression (*cyclin A*, *cyclin B*, *cyclin C*, *cyclin D*, *cyclin*

E, *Caspase9*, *Caspase2*, *p53*, *p21*, *AKT*), total RNA was extracted using RNX-PLUS reagent, and quality was confirmed by A260/A280 ratio (>1.9). cDNA was synthesized from 500 ng/20 μ L of RNA using a miScript II RT kit without interruption (CinnaGen). qRT-PCR was performed with SYBR Green master mix on a MyGo Pro instrument. Cycling conditions included an initial denaturation at 95 °C, followed by 40 cycles of amplification (95 °C for 15 s and 60 °C for 45 s and 48 °C for 30 s). Primers were designed from the Primer 3 and Integrated DNA Technologies databases, ordered from a commercial supplier (Sinaclon), verified for specificity using NCBI BLAST software, and are provided in Table 1. The mean Cq values of Gapdh was used as the reference genes for normalization. Threshold cycle (Ct) values were obtained, and the relative expression of target genes was analyzed using the $2^{-(\Delta\Delta Ct)}$ ($2^{\Delta\Delta Ct}$) method as described by Livak and Schmittgen (2001) (Livak & Schmittgen, 2001). The $\Delta\Delta Ct$ values were calculated using the formula: $\Delta\Delta Ct$ (target) = (Ct_{target} – Ct_{ref}) – (Ct_{control} – Ct_{ref}), and for controls: $\Delta\Delta Ct$ (control) = (Ct_{control} – Ct_{ref}) – mean (Ct_{control} – Ct_{ref}).

Table 1 The primer sequences.

Gene	Product Length (bp)	Length	Melting Temperature (Tm)	Designed on exons	Primer Sequence (5'-3')
<i>Gapdh</i>	96	21	57.97	7,8	F: GTGGTCTCTCTGACTTCAAC
		22	58.09		R: GGAAATGAGCTTGACAAAGTGG
<i>AKT1-202</i>	82	18	58.33	12,13	F: CAGGTCACGTCGGAGACT
		20	58.47		R: CTTGGTCAGGTGGTGTGATG
<i>P53</i>	96	22	59.65	6,7	F: GCGTGTGGAGTATTGGATGAC
		21	58.82		R: TGGATGGTGGTACAGTCAGAG
<i>p21/CDKN1A</i>	128	22	58.87	2,3	F: GTCAGTGTCTGTACCCTTGTTG
		20	58.11		R: GGCGTTTGGAGTGGTAGAAA
<i>CASP9-201</i>	99	18	57.80	7	F: TGGCTCTGGTACGTTGA
		20	58.26		R: GAAACAGCATTAGCGACCCT
<i>CASP2</i>	129	20	58.06	9,10	F: GGAGAGTGATGCCGGTAAAG
		21	57.95		R: ATGTACCAGGAACCTCGTTTG
<i>CCND1 cyclin D1</i>	126	20	57.81	1-2	F: AATGTGTGCAGAAGGAGGTC
		19	58.01		R: AGCGGTCCAGGTAGTTTCAT
<i>CCNC cyclin C</i>	142	21	59.05	10-11	F: GTTTGCTGAGCTTTCTGTGGA
		20	58.48		R: AGGTGGTTTTGGTTTTGGCA
<i>CCNA2 cyclin A2</i>	124	21	57.25	1-2	F: GCTGCTCCAACAGTAAATCAG
		21	56.66		R: GGTATGGGTACAGCATCTATCA
<i>CCNE1 cyclin E1</i>	93	21	57.87	11-12	F: ACACAACATACAGACCCACAG
		22	57.03		R: A GGAGAAGCCCTATTTTGTTCAG
<i>CCNB1 cyclin B1</i>	101	22	58.04	4,5	F: GCAGTAAATGATGTGGATGCAG
		21	57.61		R: TGCTCTTCTCAAGTTGTCTC

Evaluation of Apoptosis

Apoptosis in HT-29 cells was measured using Annexin V-FITC/PI staining (BD Biosciences), following the manufacturer's instructions. Cells (1×10^6 /well) were treated with *B. fragilis* supernatant and lysate at concentration of 10^8 CFU/mL for 24 and 48 hours. Cells treated with BHI-containing RPMI1640 medium served as the negative control. Following treatment, cells were harvested, washed with cold PBS, and resuspended in binding buffer. Annexin V-FITC (5 μ L) and PI (5 μ L) were added to each tube and incubated for 15 minutes at room temperature. After adding 400 μ L of binding buffer, samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA). The apoptotic index (AI) was calculated using the formula: AI (%) = $100 \times$ apoptotic cells / total cells (with apoptotic cells being Annexin V-positive).

Cell Cycle test

To analyze the cell cycle, HT-29 cells (1×10^6 /well) were cultured in 6-well plates and treated with 10% *B. fragilis* supernatant and lysate (10^8 CFU/mL) for 24 and 48 hours. Cells treated with BHI-containing RPMI1640 medium served as the negative control. Post-treatment, cells were washed with PBS and fixed in 70% cold ethanol for 1 hour at 4 °C. After fixation, cells were centrifuged, resuspended, and stained with PI/RNase A solution (50 μ g/mL each) at 37 °C for 30 minutes in the dark. Flow cytometric analysis was performed in triplicate using a BD FACSCalibur system.

Statistical Analysis

Data were analyzed using GraphPad Prism 9.0. Comparisons between two groups were assessed by unpaired Student's *t*-test. Multiple group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparisons test. Results are presented as mean $\pm$ SEM, with significance set at $p < 0.05$. Symbols indicate levels of significance: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

RESULTS

B. fragilis Supernatant and Lysate (*B.f Su&Ly*) Reduces HT-29 Cell Viability in a Time- Dependent Manner

To evaluate any time-dependent cytotoxic effect of *B.f Su&Ly* on HT-29 cells, MTT assays were performed following treatment with 10^8 CFU/mL concentration for 24 and 48 hours.

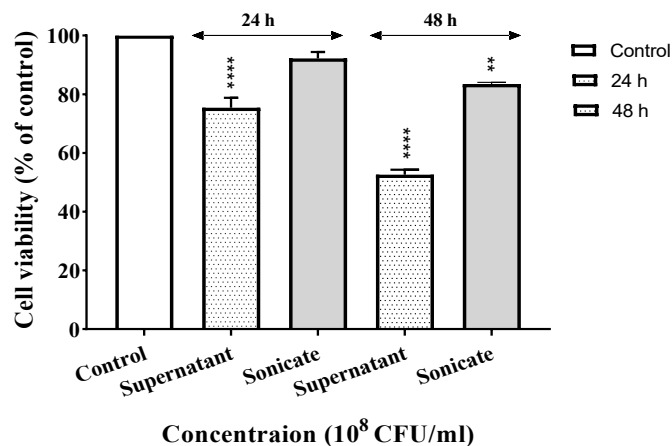


Figure 1 MTT assay results showing the effect of *B. fragilis* Supernatant and Lysate (*B.f Su&Ly*) treatment on HT-29 cell viability at 24 and 48 hours. A clear time-dependent decrease in cell survival was observed after treatment with 10^8 CFU/mL *B.f Su&Ly* compared to the untreated control. Data are presented as percentage viability relative to control and analyzed using Tukey's multiple comparisons test. Bars represent the mean $\pm$ SEM. Statistical significance is indicated as follows: * $p < 0.05$ (), ** $p < 0.01$ (), *** $p < 0.001$ (), and **** $p < 0.0001$ (****).

As shown in Fig 1, both the *B. fragilis* supernatant and lysate reduced HT-29 cell viability in a time-dependent manner, with statistically significant effects observed ($p < 0.05$). Notably, a significant reduction at 24 hours wasn't only seen with the lysate. Overall, both treatments impaired cancer cell viability, though the supernatant consistently produced a stronger effect than the lysate at both time points.

B. fragilis Supernatant and Lysate (*B.f Su&Ly*) Modulates Apoptosis-Cell Cycle-Related Gene Expression

To explore the potential role of *B. fragilis* supernatant (Su) and lysate (Ly) in inducing apoptosis and cell cycle arrest, the expression levels of ten key regulatory genes were analyzed following 24-hour exposure to 10^8 CFU/mL. As shown in Fig 2, the expression of five cyclins (A, B, C, D, and E) was evaluated. All cyclins exhibited a noticeable decrease post-treatment, with statistically significant changes observed for all except cyclin C, which showed a non-significant decrease. Fig 3 highlights the expression of five apoptosis-related genes: *Caspase-9*, *Caspase-2*, *p53*, *p21*, and *AKT*. Compared to untreated controls, *Caspase-9*, *Caspase-2*, *p53*, and *p21* were upregulated, while the anti-apoptotic gene *AKT* was downregulated. Given the role of AKT signaling in promoting cell survival and therapy resistance, its suppression further supports the pro-apoptotic potential of these treatments.

Taken together, these results suggest that both the supernatant and lysate of *B. fragilis* modulate key molecular pathways involved in apoptosis and cell cycle regulation in HT-29 cells. However, the supernatant appeared to induce more pronounced changes, which may underlie its stronger apoptotic and cell cycle-inhibitory effects compared to the lysate.

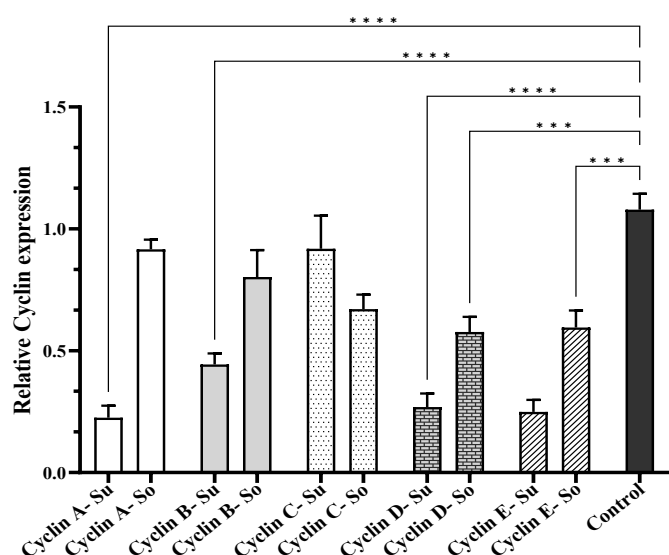


Figure 2 The effect of *B. fragilis* supernatant and lysate on relative mRNA alterations of cyclin A, cyclin B, cyclin C, cyclin D, cyclin E. The expression changes are mentioned in diagrams as fold change. Data are represented as means ($\pm$ SEM) of three independent experiments. Asterisks indicate statistically difference ($P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: *** and $p < 0.0001$: ****).

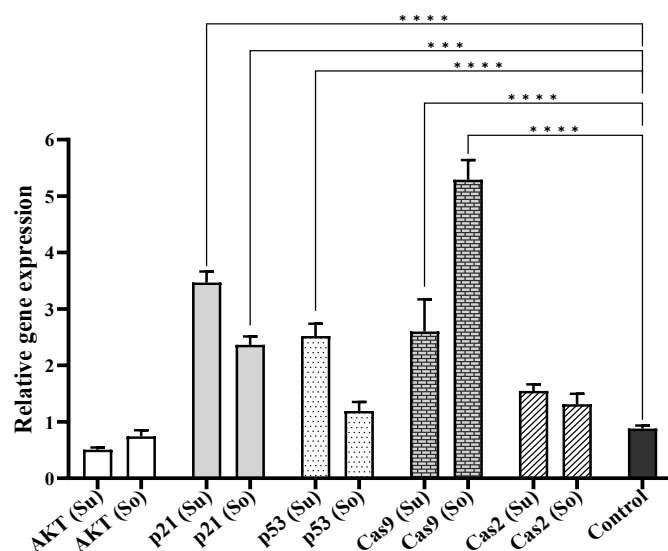


Figure 3 The effect of *B. fragilis* supernatant and sonicate on relative mRNA alterations of *Caspase-9*, *Caspase-2*, *p53*, *p21*, and *AKT*. The expression changes are mentioned in diagrams as fold change. Data are represented as means ($\pm$ SEM) of three independent experiments. Asterisks indicate statistically difference ($P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: *** and $p < 0.0001$: ****).

Time-Dependent Induction of Apoptosis in HT-29 Cells by *B. fragilis* Supernatant and Lysate (*B.f Su&Ly*), with Enhanced Efficacy Observed in the Supernatant

Annexin V/PI staining was used to determine if *B.f Su&Ly* could enhance the apoptosis of HT-29 cells. As shown in Fig 4, *B.f Su&Ly* treatment significantly increased apoptotic cell populations compared to untreated control. On average, over the 3 repetitions, *B. fragilis* supernatant and lysate increased apoptotic index (AI%) by 20% and 7% respectively at 24 h. However, apoptosis increased more by 29% and 13% respectively after 48 hours. Interestingly, our findings demonstrated that the *Bacteroides* supernatant induced a more pronounced apoptotic in HT-29 cells compared to the bacterial lysate.

Sub-G1 Accumulation and Cell Cycle Arrest in HT-29 Cells Induced by *Bacteroides fragilis* Supernatant and Lysate (*B.f Su&Ly*): A Flow Cytometric Analysis

Given the essential roles of various cyclins in regulating cell cycle transitions—and in light of the observed downregulation of cyclin expression alongside increased apoptosis following treatment with *B. fragilis* supernatant and lysate—we further investigated their impact on cell cycle progression using flow cytometry, followed by statistical analysis (One-Way ANOVA). A 24-hour exposure to the *B. fragilis* supernatant led to a significant accumulation of cells in the sub-G1 phase, accompanied by a reduction in other cell cycle phases. Extending the treatment to 48 hours further increased the sub-G1 population, indicating more cell death. A similar trend was observed with the *B. fragilis* lysate, which also elevated the sub-G1 phase ratio at 24 hours, with a more notable effect after 48 hours. However, the increase in sub-G1 phase induced by the lysate was consistently less pronounced than that induced by the supernatant, indicating a stronger apoptotic effect of the supernatant on HT-29 cells.

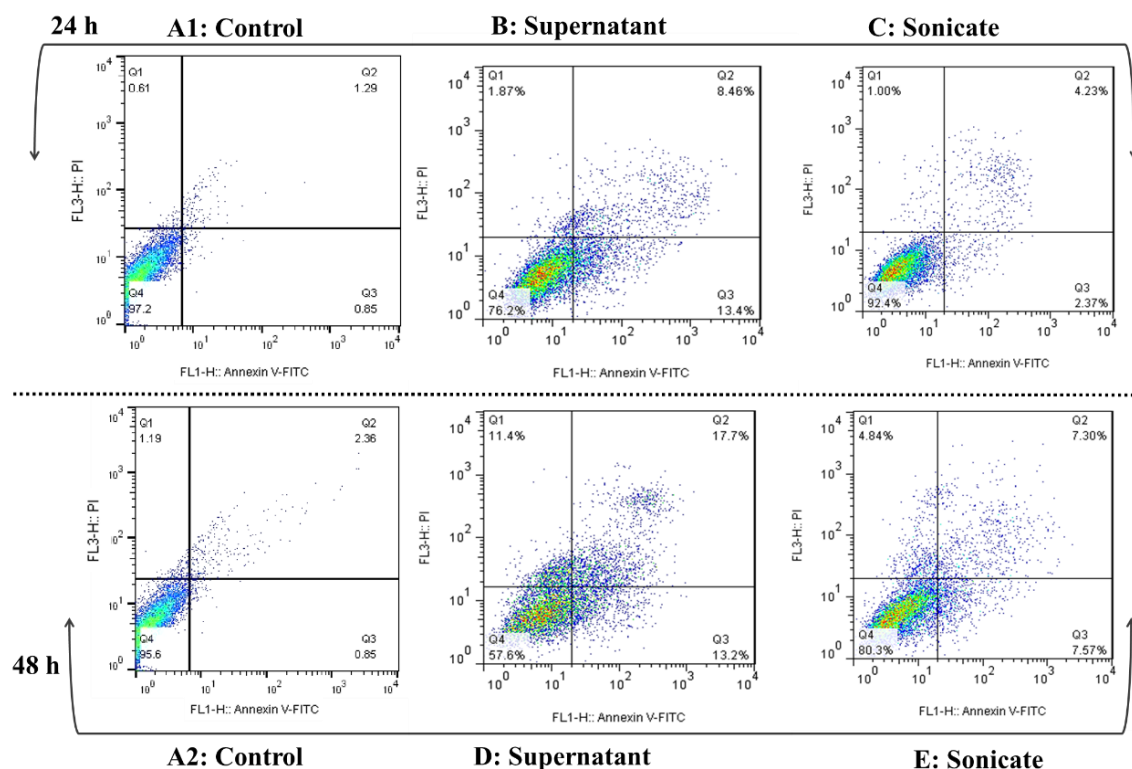


Figure 4 Flow cytometry dot plots illustrating the apoptotic effect of *B. fragilis* supernatant and lysate treatment on HT-29 cells. A1: Control (24h) and A2: Control (48h) exhibited minimal apoptosis, while cells treated with *B. fragilis* supernatant and lysate for 24 hours (B and C) and 48 hours (D and E) showed a marked increase in apoptotic populations. Dot plots display live (Q1), early apoptotic (Q2), late apoptotic (Q3), and necrotic cells (Q4). Treatment with *B. fragilis* supernatant and lysate significantly enhanced apoptosis in HT-29 cancer cells in a time-dependent manner, with the supernatant producing a more pronounced effect compared to the lysate and untreated control.

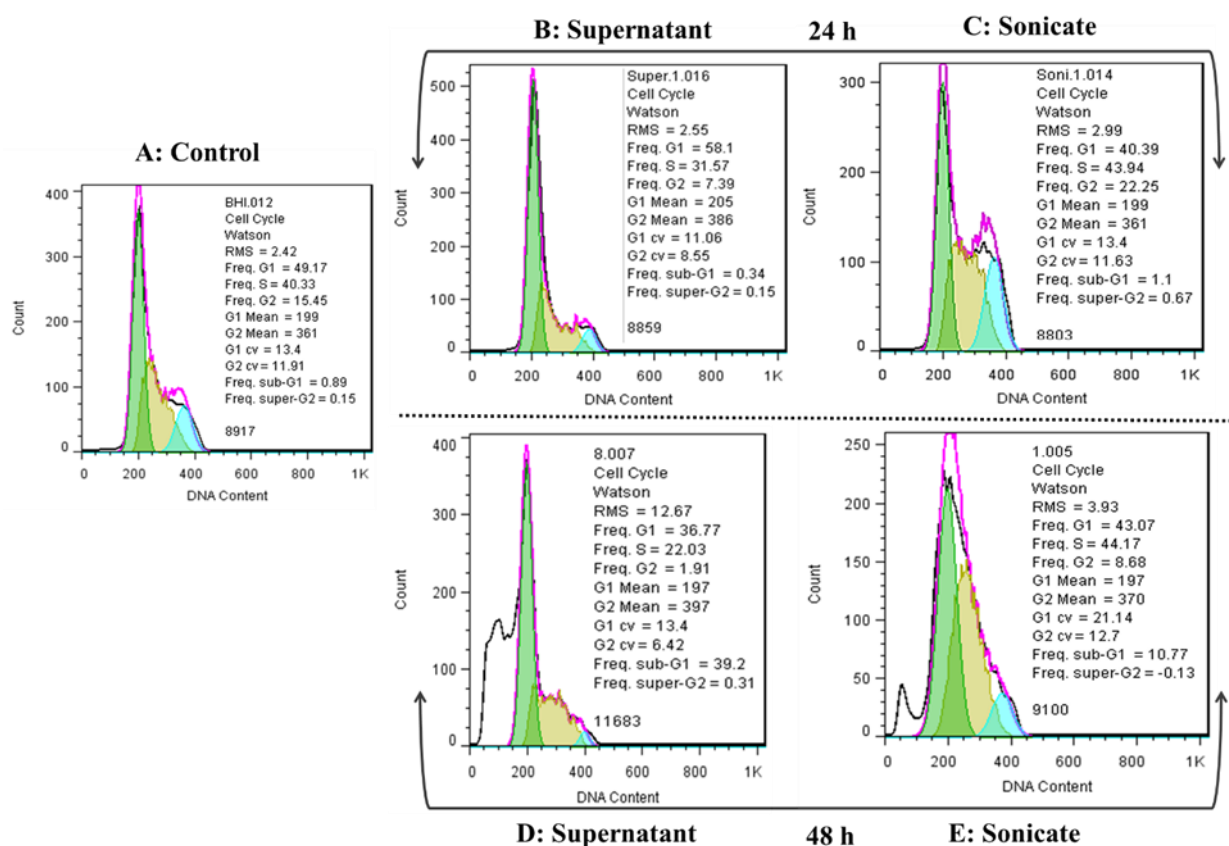


Figure 5 Flow cytometry analysis of cell cycle distribution in HT-29 cells following treatment with *B. fragilis* supernatant and lysate for 24 and 48 hours. (A) Representative histogram of control cells cultured in BHI-supplemented RPMI1640 medium. Panels (B) and (C) show cell cycle profiles after 24-hour treatment with *B. fragilis* supernatant and lysate, respectively, while panels (D) and (E) illustrate the corresponding distributions following 48-hour treatment.

DISCUSSION

The gut microbiome plays a pivotal role in regulating intestinal function, modulating inflammation, and influencing cancer development. Among its dominant members, the phylum Bacteroidota—particularly the genus *Bacteroides*—has gained considerable attention. Although they regarded as a potential pathogen and show potential in managing various health conditions, including gut disorders, behavioral issues, and cancer, but *Bacteroides fragilis* has recently emerged as a candidate for next-generation probiotics. This is due to their strong interactions with the host immune system and their ability to influence metabolic pathways in the gut. Advances in genomic tools, such as metagenome-assembled genome (MAG) binning highlights their role in immune regulation, inflammation control, and disease prevention. Their safety, therapeutic potential, and effectiveness are under review by the European Commission for potential use in food applications (Cang et al., 2024; M. A. Tufail & R. A. Schmitz, 2025).

Growing interest surrounds the role of gut microbiota in cancer therapy. An analysis of patients undergoing oral nutritional supplements combined with probiotics, showed the probiotic group improvements in liver enzymes (ALT, AST), reduced endotoxin levels, and a healthier gut microbiota profile—marked by increased beneficial bacteria like *Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Bacteroides fragilis*, along with decreased harmful microbial species such as *Escherichia coli* and *Enterococcus faecalis*. These results suggest *Bacteroides fragilis* as a beneficial bacterial community that may help support gut and liver health during chemotherapy (Li et al., 2025).

In our study, we demonstrate that both the supernatant and lysate of *B. fragilis* can modulate key molecular pathways involved in colorectal cancer. Our study provides evidence that treatment with *B. fragilis* led to the upregulation of pro-apoptotic genes and downregulation of cyclins, thereby interfering with cell cycle progression and promoting apoptosis. These findings suggest a possible role for *B. fragilis* in reducing colorectal carcinogenesis.

Studies have shown that *Bacteroides ovatus* and *Bacteroides fragilis* ATCC25285 can help reduce inflammation and ease symptoms of colitis. Similarly, *Bacteroides thetaiotaomicron* has demonstrated strong anti-inflammatory effects, notably improving the integrity of the mucosal barrier, limiting the invasion of harmful pathogens, and reducing colon inflammation in experimental models of Crohn's disease. Cell-free supernatants derived from the commensal strains, *Bifidobacterium bifidum* and *Bacteroides fragilis*, demonstrated notable anticancer activity in both *in vitro* and *in vivo* breast cancer models. These supernatants effectively suppressed tumor growth by inducing necrosis and inhibiting both cell proliferation and angiogenesis (He et al., 2023; Karami et al., 2023; Luo et al., 2025; Muhammad Aammar Tufail & Ruth A. Schmitz, 2025).

Consistent with these findings, our MTT assays provides evidence that both the *B. fragilis* supernatant and lysate (*B.f Su&Ly*) reduced HT-29 cell viability in a time-dependent manner, though the supernatant consistently produced a stronger effect than the lysate at both time points. Annexin V/PI staining also demonstrated that *B.f Su&Ly* significantly enhanced apoptosis, with the supernatant producing a more pronounced effect compared to the lysate and untreated control.

Existing literature suggests that oral administration of *B. salyersiae* CSP6, *Bacteroides plebeius* and *Bacteroides fragilis* provides significant protection against colitis and colitis-associated colon cancer in mice. Treatment with CSP6 or *B. plebeius* alleviated mucosal damage, corrected gut dysbiosis, reduced tumor formation, supports the production of protective metabolites, and enriched beneficial species like *Bifidobacterium*. Additionally, these two species plus elevated levels of anti-inflammatory and tumor-suppressive metabolites and had a strong therapeutic benefit (Chen et al., 2025; Dai et al., 2024; Geng, Wang, Wu, Cao, & Liu, 2023).

Furthermore, Short-chain fatty acids (SCFAs), such as butyrate, are metabolic byproducts produced by gut microbiota during the fermentation of dietary fiber and are known for their anti-cancer properties. *Lactobacillus plantarum* strain S2T10D demonstrated notable anti-proliferative effects on HT-29 colorectal cancer cells. These effects were attributed to its butyrate-producing capacity, which led to the downregulation of key cell cycle regulators, cyclin B1 and cyclin D1, and resulted in cell cycle arrest at the G2/M phase (Wu et al., 2024).

Our study demonstrated cell cycle analysis of HT-29 cells treated with *B. fragilis* supernatant resulted in a marked increase in the sub-G1 cell population after 24 hours, suggesting the induction of apoptosis. This effect became more pronounced after 48 hours of exposure indicating more cell death. A comparable, though less intense pattern was observed with the *B. fragilis* lysate, which also increased the sub-G1 ratio over time. Remarkably, the apoptotic effect of the supernatant was consistently stronger than that of the lysate, highlighting its greater potency in promoting cell death in colorectal cancer cells.

Collectively, these results highlight the functional complexity of bacterial secretions and point to the presence of specific, bioactive compounds in the supernatant that may selectively activate signaling pathways to inhibit tumor cell proliferation. In contrast, the lysate—comprising a mixture of intracellular contents such as cell wall components released through bacterial lysis—appears to induce a more generalized cellular stress response and trigger autophagy-related mechanisms, which could potentially interfere with the apoptotic response.

The progression of the cell cycle is controlled by a highly coordinated network of regulatory proteins, including cyclins. Recent studies have shown that combining

cyclin inhibitors with agents targeting the PI3K/AKT signaling pathway can produce synergistic anti-cancer effects. These combinations appear to enhance apoptotic responses through the activation of key mediators such as caspase-9, p21, and p53, suggesting a promising strategy for improving therapeutic efficacy (Lim et al., 2021; Pellarin et al., 2025).

To further investigate the potential of *Bacteroides fragilis* supernatant and lysate in triggering apoptosis and cell cycle arrest, we examined the expression of ten key regulatory genes after 24 hours of treatment at a concentration of 10⁸ CFU/mL. All five tested *cyclins* (*A*, *B*, *C*, *D*, and *E*) showed reduced expression following treatment. Additionally, pro-apoptotic markers including *Caspase-9*, *Caspase-2*, *p53*, and *p21* were upregulated, while the anti-apoptotic gene *AKT* was downregulated compared to the untreated control. Overall, these findings indicate that both the supernatant and lysate of *B. fragilis* influence essential cell cycle and apoptosis-involved genes, emphasizing the point that supernatant had a more pronounced impact, which may explain its stronger ability to induce apoptosis and inhibit cell proliferation compared to the lysate. The enhanced efficacy of the *B. fragilis* supernatant engage specific signaling pathways that suppress tumor cell proliferation. This specificity underlines the potential of utilizing bacterial secretomes and possibly its outer membrane vesicles (OMVs) or their metabolites like PSA, as next-generation probiotics or postbiotic agents. They could help by reducing inflammation and slowing tumor growth, especially in the early stages of the disease, thereby be consider as supportive treatments alongside conventional colorectal cancer therapies.

There are still some deficiencies in this work that need further completion and betterment. One limitation of our study is that we relied on a single colorectal cancer cell line (HT-29), which may not fully represent responses in all colorectal cancers or most biological responses. Future studies should include non-cancerous intestinal epithelial cell lines to evaluate potential cytotoxic effects on healthy tissue. Additionally, incorporating immune cell lines would provide valuable insights into how *Bacteroides* extracts influence immune-related pathways, including cell viability, apoptosis, and cytokine responses. While we focused on *in vitro* analysis, complementary approaches such as *in vivo* or immune-competent models are needed to evaluate therapeutic safety, efficacy, and immune responses. Moreover, our experiments used whole supernatant and lysate preparations. Further purification or fractionation of these bacterial extracts would help identify the active components and better distinguish the functional differences between the supernatant and lysate.

CONCLUSION

In summary, our findings show that both the supernatant and lysate of *Bacteroides fragilis* can influence key molecular pathways related to cell cycle regulation and apoptosis in colorectal cancer cells. While both treatments reduced HT-29 cell viability and promoted apoptotic activity, the supernatant consistently produced stronger effects. We believe that specific bioactive compounds within the supernatant may more effectively target tumor cell survival mechanisms, highlighting its potential as a promising candidate for anti-cancer strategies.

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Competing interests: All authors – none to declare.

Funding: None to declare.

Authors' contributions: Aras Rafiee: wrote the manuscript, analyzed the data, designed and supervised the project.

Mona Bagheri and Yeganeh Marandi: carried out the experiments, helped in data acquisition, analysing the data and was in charge of overall planning. Zahra Shahi, Seyed Ataollah Sadat Shandiz involved in Technical or Material Support. All authors provided critical feedbacks, discussed the results and commented on the manuscript.

List of abbreviations: CRC: colorectal cancer; BA: *Bacteroides fragilis*; *B. fragilis* Supernatant and Lysate (*B.f Su&Ly*);

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