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Original Article

Bacteroides Fragilis Supernatant Plays Anti-Viability Roles Accompanied by Apoptosis and Cell Cycle Arrest via P62/Caspase8/ Bax/Fas Pathway in Colorectal Adenocarcinoma Cell Line

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Abstract

From two classes of *Bacteroides fragilis, enterotoxigenic B. fragilis (ETBF) is associated with colorectal cancer (CRC), yet several non-toxigenic B. fragilis (NTBF) confers powerful health benefits to the host* and *may be potential probiotic. In this study, the HT-29 cell line was treated with the* supernatant of *NTBF* strain ATCC-23745. Then, the expression level of mTOR /p62/Caspase8/Bax proposed signaling pathway and cell viability, cell apoptosis, and cell cycle progression were determined using Real-time PCR and flow cytometry, respectively. We found that the *B. fragilis* supernatant inhibited cell proliferation and increased cell apoptosis in a dose- and time-dependent manner. Further, the arrest of the HT-29 cells at the G1 and sub-G1 phases also signified apoptotic cell death after 24 and 72h. The gene expression study revealed that the supernatant significantly up-regulated the Caspase8/Bax/Fas-mediated apoptosis signaling while suppressing the anti-apoptotic mTOR and p62 expression. Our findings suggest that the NTBF ATCC-23745 strain may be a potential probiotic and can be used in CRC treatment.

Keywords: Apoptotic death; Colorectal cancer; Next generation probiotic; Nontoxigenic *Bacteroides fragilis*; Signaling pathway.

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1. Introduction

Major Gram-negative bacteria discharge major virulence components such as bacterial amyloids, enzymes, endotoxins and exotoxins, cardiolipins (CLs), and small non-coding RNAs (sncRNAs) [1]. These components can induce or repress multiple activities, including apoptotic cell death, intracellular reactive

oxygen species (ROS), autophagy, cell cycle, and cell proliferation [2].

Bacteroides fragilis is a commensal gut bacterium of humans, known to be predominantly composed of either Enterotoxigenic *Bacteroides fragilis* (ETBF) strains or non-toxigenic *B. fragilis* (NTBF) strains. ETBF strains can produce a proteolytic enterotoxin named *B. fragilis* toxin (BFT). BFT is a virulence factor for enteritis and colorectal cancer (CRC) [3]. Furthermore, BFT activates Ras homolog enriched in the brain (RHEB)/mammalian target of the rapamycin (mTOR) pathway and ultimately results in CRC tumor growth [4].

Although the ETBF strains have recently been shown to affect colorectal cancer, NTBF strains are now proposed as beneficial therapeutic microbes to moderate colon inflammation, preventing colitis and protecting the gut epithelia from colonization by pathogenic bacteria [5, 6]. Co-colonization of mice with NTBF and ETBF strains shows a competitive exclusion of ETBF by NTBF strain, which reduces BFT transcript in the feces and protects the host against toxin-induced colitis [7]. It has also been demonstrated that human commensal *B. fragilis* can generate simplified polysaccharide A (PSA) and protects against colon tumorigenesis in a mouse model of colitis-associated CRC [8, 9]. Vetizou *et al*. have reported that antibodies targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) can be used as cancer immunotherapy. The therapeutic efficacy of CTLA-4 blockade has been enhanced by microbiota consumption (B. thetaiotaomicron or B. fragilis), improving antitumor immune responses [10]. Furthermore, *Bacteroides* species produce a short-chain fatty acid named propionate in response to indigestible carbohydrates in the diet. The propionate derived from *Bacteroides thetaiotaomicron* (BT) supernatant has an anticancer effect at the epigenetic level and induces colon cancer apoptosis [11].

As mentioned above, mTOR signaling mainly involves tumor initiation and progression [5]. Furthermore, p62/SQSTM1 (p62) requires mTOR activation and may be a potential indicator of determining colon cancer progression [12].

On the other hand, in the extrinsic apoptotic pathway, cell-surface death receptors such as FAS trigger apoptosis through a death-inducing signaling complex (DISC). Then, the DISC recruits the apical protease caspase-8. Efficient activation of caspase-8 leads to stimulation of downstream effector caspase-3/7 to initiate cell apoptosis [13]. Moreover, caspase eight up-regulation and p62 deficiency can also directly or indirectly activate pro-apoptotic BCL2-associated X protein (BAX) at the mitochondrial membrane [14, 15].

Finally, the differences in the function of ETBF and NTBF strains suggested to the authors that NTBF might represent a useful model for the study of CRC treatments by modulation of the p62/Caspase8/Bax/FAS hypothetical signaling pathway. Another purpose of our study was to determine whether *non-toxigenic B. fragilis* supernatant *(*NBFS) can induce apoptosis and cell cycle arrest in HT-29 cells, which is important for developing new treatments for CRC.

2. Materials and Methods

2.1. Bacterial culture and preparation of supernatant extract

The freeze-dried form of NTBF *B. fragilis* (ATCC 23745) was obtained from the Pasteur

Institute of Iran. The culture was accomplished in an anaerobe chamber (80% N2, 10% H2, and 10% CO2). 1.0 mL of brain heart infusion (BHI) broth (Merck) was poured on a lyophilized bacterial pellet to rehydrate the entire content, then incubated for 15 min at 37°C after pipetting for 10 sec. This aliquot was transferred into a tube containing 9 mL of BHI broth. Several drops of the suspension were inoculated on BHI agar to check for purity. The cultures were incubated for 16 h. The optical density of culture growth was obtained at 600 nm (OD600) and harvested in an exponential phase. Bacterial cells were centrifuged at 5,000 g for 5 min at 4°C and filtered through a 0.22-μm filter membrane (Bioyfil) to separate the supernatant [16].

2.2. Ht-29 cell culture

A colorectal cancer cell line (HT-29) was purchased from the National Cell Bank of Iran, cultured in RPMI 1640 medium containing 1% (v/v) penicillin-streptomycin antibiotics supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated in flasks in a humidified atmosphere with 5% CO2 and 95% air at 37˚C in a CO2 incubator (HEPA class 100; Thermo Fisher Scientific, Inc).

2.3. MTT assay

The effect of NBFS on the growth of HT-29 cells was assessed by MTT cell growth assay. Briefly, 5 $\times 10^4$ cells were seeded in 96-well plates containing 100 μL of complete cell culture medium overnight. Then, cells were treated with 10% (10^8 CFU/ml) and 100% (10^9 CFU/ml) concentrations of NBFS. Negative controls were treated with an equal volume of RPMI-1640 medium. After 24 and 72 h of incubation, the media was removed, and cells were treated with 20 μl of 0.5 mg/ml MTT solution (Sigma). The plates were incubated for one hour at 37 °C in an incubator. The liquid was then removed, and 100 μL of DMSO was added to each well to solubilize formazan blue crystals. Following an incubation of 20 min, the absorbance was measured at 570 nm using a plate reading spectrophotometer BIO RAD®). The growth inhibition percentage of cells was calculated using the following equation: % Cell viability = (Test OD – blank OD / Negative control OD – blank OD) × 100.

2.4. Quantitative Real-Time PCR

The expression of the mTOR, p62, caspase8, and BAX genes was detected by RT-qPCR. Following the manufacturer's protocol, total RNA was extracted with RNX-PLUS reagent (Cinnagen, Iran). The integrity and concentration of the RNA were determined by measuring the optical density at A260 nm/A280 nm with an Epoch Microplate Spectrophotometer (BioTeck), acceptable when the ratio was >1,8.

Total RNA (1μg) was reverse transcribed using the easy cDNA synthesis kit (Parstous, Iran) according to the manufacturer's procedures. Real-time PCR amplifications were performed using RealQ Plus 2x Master Mix Green kit (Ampliqon) on a MyGo Pro real-time PCR instrument (IT-IS Life Science). The three-step qRT-PCR program included a 95°C denaturation step for 15s followed by 40 cycles of 95°C for 15s and, 60°C for 45s and 72°C for 30 s. Primers were designed using IDT and Primer 3 online software. The primer sequences are listed in **Table 1**.

Table 1: The primer sequences of p62, mTOR, Caspase8, Bax, Gapdh, and Hprt1 genes.

		Primer sequence (5'->3')	Tm	Product length bp
P62	F	TGATTGAGTCCCTCTCCCAGAT	60.02	100
	R	GCCGCTCCGATGTCATAGTT	60.25	
mTOR	F	CTGATTCTCACAACCCAGCG	58.92	129
	R	ATCATCCCGATTCATGCCCT	58.93	
Caspase 8	F	CGGGGATACTGTCTGATCATCA	59.11	128
	R	TCAAAGGTCGTGGTCAAAGC	58.69	
Bax	F	TTTCTGACGGCAACTTCAACTG	59.65	127
	R	TCCAATGTCCAGCCCATGA	58.60	
Gapdh	F	GTGGTCTCCTCTGACTTCAAC	57.97	96
	R	GGAAATGAGCTTGACAAAGTGG	58.90	
Hprt1	F	AAGGGTGTTTATTCCTCATGGAC	58.40	105
	R	AGCACACAGAGGGCTACAA	58.55	

Gapdh and Hprt were used as housekeeping (reference) genes for normalization. The threshold cycle (Ct) value was recorded, and the Livak method ($2^{-\Delta\Delta CT}$) was used to analyze the relative changes in gene expression [17], which is described as follows:

$\Delta\Delta C_{T(target)}=(C_{T\ \text{target}}-C_{T\ \text{ref}})-(C_{T\ \text{control}}-C_{T\ \text{ref}})$ and $\Delta\Delta C_{T(control)}=(C_{T\ \text{control}}-C_{T\ \text{ref}})_{\text{control}}-\text{mean}\ (C_{T\ \text{control}}-C_{T\ \text{ref}})$.

2.5. Apoptosis Detection

According to the manufacturer's protocol, cellular apoptosis was determined using the Annexin-V Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences). HT-29 cells were seeded in 6–well culture plates at a density of 1×10^6 cells/well. After 48 h incubation, cells were treated with the NBFS at 10^8 CFU/ml and 10^9 cfu/ml for 24 and 72 h. BHI containing RPMI1640 medium was used as the negative control. Briefly, Cells were suspended in 1X Binding Buffer. First, FITC Annexin V (5 µl) and then propidium iodide (PI) (5 µl) were added to the cells. Finally, the test tube was washed with 1x PBS, and apoptotic cells were detected by a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA, USA). The results were analyzed by FLOWJO software (version 7.6, USA). The apoptotic index (AI) was calculated by dividing the percentage of apoptotic cells.

($AnnexinV^+$) by the total percentage of cells in the sample ($AnnexinV^+$ plus $AnnexinV^-$). The following formula was used to calculate AI: AI (%) = 100 × apoptotic cells/total cells [18].

2.6. Cell Cycle Analysis

HT-29 cell line at 1×10^6 cells was cultured in 6-well plates in the presence of the NBFS at a suspension concentration of 10% (10^8 cfu/ml) for 24 and 72 h. BHI containing RPMI1640 medium was used as the negative control. After treatment, the cells were washed twice with PBS, and then the cells were resuspended and transferred to a 5 ml tube containing cold 70% ethanol at 4°C for 1h. After ethanol fixation and

twice washing with PBS, the cells were stained with propidium iodide (PI) solution (PBS containing 50 µg/ml PI, 50 µg/ml RNase A) at 37˚C for 30 min in the dark. Finally, flow cytometry was performed in triplicate for each experiment using the BD FACSCalibur system (BD Biosciences, USA).

2.7. Statistical analysis

All statistical analysis was performed using Graphpad Prism 7.0 (Prism 7.0 Graphpad Software Inc., La Jolla, USA). An Independent sample t-test (unpaired Student's t-test) was conducted to compare two conditions. A one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test evaluated the statistical differences among the groups. All data were presented as the mean ± SEM. The results were considered statistically significant when the P value was $p<0.05$. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ and ****$p<0.0001$.

3. Results and Discussion

3.1. NBFS inhibits the viability of HT-29 cells in a time- and dose-dependent manner

HT-29 cells were treated with 10^8 and 10^9 cfu/ml concentrations of NBFS for 24 and 72h to investigate its effect on cell survival using the MTT assay. As shown in **Figure 1**, NBFS has an antiproliferative and cytotoxic effect on HT-29 cells in a time- and dose-dependent manner. The 10^8 and 10^9 cfu/ml concentrations of NBFS treatment decreased the viability of cells to 83.4 % and 76.8 %, respectively, which substantially decreased to 51.5 % and 33.1% after 72h. In this regard, the Inhibitory concentration (IC50) of NBFS was approximately 10^8 cfu/ml after 72 hr related to HT29 cells. All values and results are presented based on the mean ± SEM of three independent repetitions.

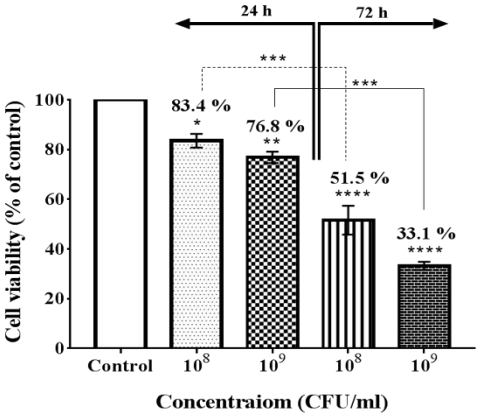

Figure 1. The MTT assay results: The Effect of 24 and 72 h exposure to NBFS treatment at 10^8 and 10^9 cfu/ml on the viability of the HT29 cell line. As can be seen, the inhibition of cell survival increases in a dose-time-dependent manner. The results are reported as survival percentages compared with the control and were considered statistically significant when Tukey's multiple comparisons test P value was $p<0.05$. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ and ****$p<0.0001$. The lines drawn at the top of the charts indicate a significant comparison between the different groups.

3.2. Apoptotic induction in HT–29 Cells treated with NBFS

The effect of 10^8 and 10^9 cfu/ml NBFS treatment on the induction of apoptosis in HT29 cells was investigated using flow cytometric analysis. The results using annexin V/PI double staining indicated that NBFS significantly increased the proportion of apoptotic cells at all-time points compared with the control. Examples of the flow cytometry results on the HT29 cells, shown as dot plots, are presented in **Figure 2**.

The apoptotic index (AI) of HT29 cells was 17 % and 19% at 10^8 cfu/ml after 24 and 72, respectively. At 10^9 cfu/ml, AI was 39% and 78% after 24 and 72, respectively.

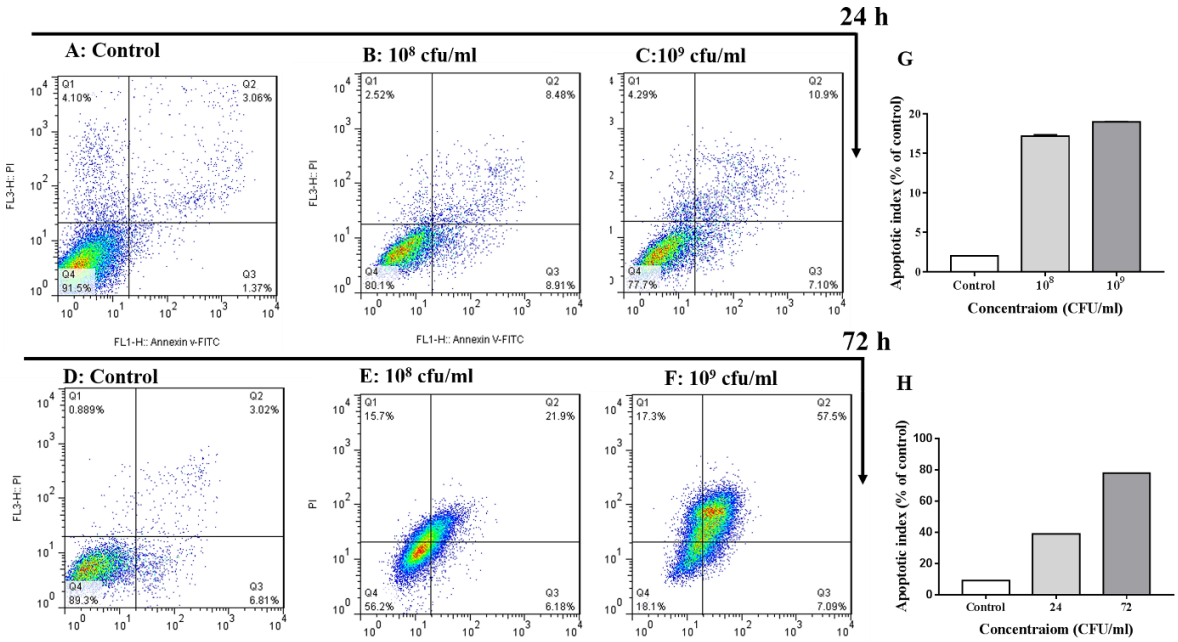

Figure 2. Flow cytometry dot plots representing the apoptosis-inducing effect of NBFS on HT-29 cells. A: Control (24h), D: Control (72h), and B and C are treated cells at 10^8 and 10^9 cfu/ml after 24 h, respectively. E and F are treated cells at 10^9 cfu/ml after 72 h. G and H show the apoptotic index (AI) of HT29 cells after 24 and 72, respectively. The dot plots show non-apoptotic cells in Q1 (AnnexinV/PI $^{-/-}$), apoptotic cells in Q2 (AnnexinV/PI $^{+/-}$), and late apoptotic cells in Q3 (AnnexinV/PI $^{+/+}$) or necrosis in Q4 (AnnexinV/PI $^{-/+}$). The addition of NBFS significantly increased the proportion of apoptotic cells at all-time points (Figure. 2C and D). These results indicate that BFSE potentiated the apoptotic death of HT29 cells

Thereby, the AI of HT29 cells was 1.8 and 2.1 times more than the control when treated with 10^8 and 10^9 cfu/ml of NBFS, respectively, after 24h. In the same way, AI was increased to 4.3 and 8.6 after 72 h.

3.3. NBFS induces subG₁/G₁ cell cycle arrest

HT-29 cells were treated with the NBFS at 10^8 cfu/ml at 24 and 72 h and stained with PI to investigate cell cycle progression. Then, the distribution of cells in the sub-G1, G1, S, and G2 phases of the cell cycle was analyzed by flow cytometry. **Figure 3** indicated that treated cells firstly showed higher subG1 (2.8%) and G1 (58.5%) compared with the control (subG1:0.9% and G1: 53%) after 24h with a concomitant reduction at DNA synthesis in S and G2 phase.

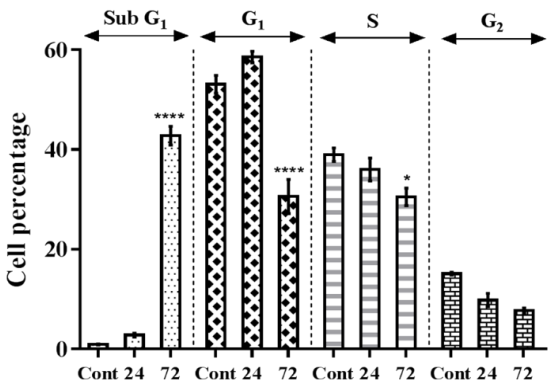

Figure 3. The effect of NBFS on the cell cycle distribution in HT-29 cells. Treated cells with 10^8 cfu/ml concentration of *B. fragilis* extract at 24 and 72 h were stained with PI to analyze the cell cycle distribution by flow cytometry. The percentage of cells in each phase of the cell cycle is calculated relative to the total number of selected cells. Control was indicated as (Cont), and treated cells were indicated 24 h (24) and 72 h (72). Each data point represents the mean (±SEM) of three independent experiments. Asterisks indicate significant difference (P<0.05*, P<0.01**, P<0.001*** and p<0001****).

Whereas the percentages of the sub-G1 phase (apoptotic cells) were significantly increased to 42.8%, and the G1 phase decreased to 30.6% after 72h ($p < 0.001$). The number of cells in the S and G2 phases decreased (72h) or was slightly similar (24h) to the levels of the control. This experiment suggested that NBFS induces apoptotic cell death in HT-29 cells after 72h.

3.4. Effect of NBFS on the expression of apoptosis-related genes

In the meantime, we revealed NBFS probably triggered SubG1 phase cell cycle arrest, which terminates in apoptotic cell death; to identify which cellular pathway induced cell apoptosis, we investigated the effect of NBFS on the expression of mTOR/ p62/ Caspase8/ Bax/FAS-mediated apoptotic genes. Gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized by the housekeeping genes Gapdh and Hrpt levels.

However, high fluctuations were observed in the expression of hprt1, which affected the results of the $2^{-\Delta\Delta Ct}$ method. Therefore, it was not used in our final calculations, and only the GAPDH gene was used as the reference gene due to fewer fluctuations (**Figure 4B**). Treatment with NBFS resulted in a clear up-regulation in the gene expression levels of Caspase8 (up to~6.3-fold) and Bax (up to~2.2-fold) and FAS (up to~2.7-fold and downregulation of mTOR (up to~2.8-fold), P62 (up to~0.7-fold), at 10^8 cfu/ml concentration after 24 h (**Figure 4A**). These results indicate that NBFS induced apoptosis signaling pathway.

Moreover, it is necessary to mention that gene expression was normalized by Gapdh and Hrpt1 housekeeping genes. However, high fluctuations in the hprt1 expression led us to use only the Gapdh to report the results. It may be due to the effect of bacterial extract on expression changes of this housekeeping gene over a wide range, which did not make it suitable for use in this research.

Enterotoxigenic *B. fragilis* produces a zinc-dependent metalloprotease termed *B. fragilis* toxin (BFT) or fragilysin, which is located on a pathogenicity island (PAI) region of the genome and leads to intra-abdominal abscess formation and colon cancer [3, 19]. The PAI enters the bacteria's chromosome from the flanking region. According to the presence of a *B. fragilis* PAI and its flanking region, *B. fragilis* strains can be classified into three patterns. (1) enterotoxigenic *B. fragilis* (ETBF) strains, or pattern I with BFT gene, PAI, and its flanking region; (2) nontoxigenic *B. fragilis* (NTBF) strains, or pattern II without the PAI and flanking region and (3) NTBF strains, or pattern III, without the PAI and with flanking region [20]. However, evidence shows that commensal nontoxigenic *Bacteroides fragilis* (NTBF) strains that do not possess a PAI region suppress colitis [21], confer powerful health benefits to the host, and have been proposed as a probiotic [22].

Therefore, we believe non-toxic *B. fragilis* is a good choice for the probiotic candidate, and we decided to choose NTBF strain ATCC-23745 (pattern III) and explore its ability to inhibit the growth of cancer cells, induction of apoptosis, and block the cell cycle whether satisfy the criteria required for probiotic bacteria.

Here, we investigated and provided evidence for the antiproliferative effects of non-toxigenic *B. fragilis* supernatant (NBFS) against colon carcinoma in vitro. Our findings show that co-incubation of HT29 cells with NBFS significantly decreased the viable cells in a concentration- and time-dependent manner.

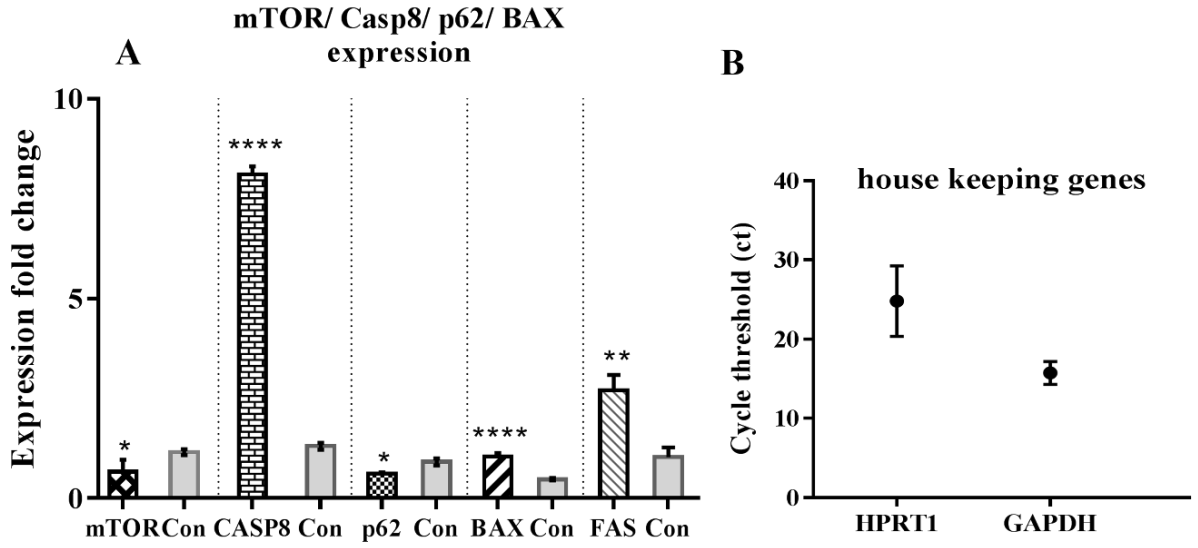

Figure 4. The effect of NBFS on relative mRNA alterations of mTOR, p62, Caspase8, Bax, FAS, Gapdh, and hprt1. A) The expression changes of mTOR/p62/Caspase8/Bax signaling in HT-29 cells are mentioned in diagrams as fold change. Data are represented as means (± SEM) of three independent experiments. Asterisks indicate statistically difference (P<0.05*, P<0.01**, P<0.001*** and p<0001****). B) Comparison of cycle threshold (ct) of two housekeeping genes Gapdh and hprt1. The GAPDH gene was used in the final computation due to less fluctuation. Error bars represent standard error.

In addition, our flow cytometry data suggested that the growth inhibitory effect of NBFS is relevant to cell-cycle arrest. After incubating cells with NBFS, a significant growth arrest in the subG1phase was observed after 72h. Arresting the cell cycle at specific checkpoints provides time to repair damage for cell integrity; however, apoptosis is initiated if the damage is too much to be repaired [23]. Besides, NBFS may delay the progression of cells through apoptotic cell death. Then, we assessed the effect of NBFS on cell apoptosis. The annexin V/PI double staining results indicated that apoptotic cells increased after 24h and 72h. In other potentially relevant work, the apoptotic factor expressed by *B. fragilis* ATCC 23745 strain as phenylacetic acid (PA) caused apoptotic cell death on Vero cells [24].

Based on the recent findings mentioned above, to further validate the mechanism by which *NBFS* induced apoptosis, we propose a hypothetical model through mTOR/ p62/ Caspase8/ Bax/FAS apoptotic signaling pathway. In support of this hypothesis, the results of the qPCR method showed us that NBFS triggers apoptosis possibly through enhanced expression of FAS, Bax, and caspase 8, which specify extrinsic apoptosis. The extrinsic signaling pathways initiate apoptosis by recruiting the death-inducing signaling complex (DISC) formed by Fas receptor, FADD (Fas-associated death domain protein), and Caspase-8, a crucial apical caspase in the extrinsic pathway [25]. DISC can activate the release of reactive oxygen species (ROS) and induce significant amounts of oxidative stress. ROS production and DISC formation disequilibrium activate apoptotic pathways [26] by elevating the levels of apoptotic proteins such as Bax and Caspase-8 [27]. The same behavior was observed in treated human leukemia cell lines with *B. forsythus* supernatant. *Bacteroides*

supernatant has been recognized to have the ability to induce apoptotic cell death, DNA fragmentation, and caspase-3 activation [28]. Likewise, manipulation of intestinal microbiota, such as consumption of lactic acid-producing bacteria, can prevent DNA damage, and the use of bacteria such as *Bifidobacterium* and *Bacteroides* can reinforce anti-carcinogenic immune therapy and, therefore, improve tumor control [29, 30].

Other genes that are involved in cancer and were also considered in this study were mTOR and p62. We also found that the expression of mTOR and p62 was significantly decreased (**Figure 5**).

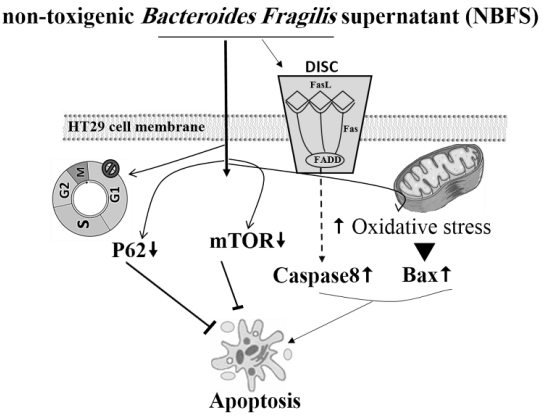

Figure 5. The schematic diagram indicates NBFS induces cell cycle arrest and apoptosis in HT-29 cells. Apoptosis is regulated through mTOR/ p62/ Caspase8/ Bax/FAS hypothetical signaling pathway. NBFS initiate extrinsic apoptosis by recruiting the transmembrane receptor Fas, the cytosolic FADD (Fas-associated death domain protein), and caspase-8, which is referred to as the Fas/FADD/caspase-8 death-inducing signaling complex (DISC). FADD activates the procaspase 8. Active caspase-8 is then released from the DISC engage apoptotic activator BAX to facilitate the release of other apoptotic proteins. Downregulation of p62 and mTOR in tumor cells also contributes to cell cycle inhibition at the sub-G1 phase.

Recent studies also revealed that mTOR and p62 proteins can activate oncogenic signaling pathways. *p62* is overexpressed in multiple forms of *cancer* and exerts its carcinogenic role through enhanced expression of *mTORC1* [31, 32].

4. Conclusion

In summary, *treating the HT-29 cell line with* nontoxigenic *B. fragilis* ATCC-23745 supernatant (*NBFS)* leads to cell cycle inhibition, initiation of apoptosis, and thus antiproliferative efficacy in a dose-time-dependent manner. The present study also revealed the apoptotic effect of *NBFS* via mTOR/p62/Caspase8/Bax/FAS signaling pathway, further suggesting that NBFS may be a therapeutic way for CRC therapy.

Acknowledgments

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Conflict of interest

The authors declare to have no conflict of interest.

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