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Bacteroides fragilis Induce Apoptosis and subG$_1$/G$_1$ Arrest Via Caspase and Nrf2 Signaling Pathways in HT-29 Cell Line

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Abstract

Background: Although the link between the human microbiome and colorectal cancer has been elucidated over recent years, its underlying mechanisms for various bacteria such as nontoxigenic Bacteroides fragilis (NTBF) are still unclear.

Methods: HT29 cells were treated with Non-toxigenic *B. fragilis* Sonicated Extract (NBF-SE), strain ATCC-23745, in the dose (10^8 and 10^9 CFU/ml) - and time (24 and 72 hours)-dependent manners. To investigate the cytotoxic impact of NBF-SE on HT29 along with apoptosis-induction and cell cycle distribution, MTT assay and flow cytometry was carried out.

Changes in the expression level of Keap1/NRF2/Caspase3/caspase10/GCLM proposed signaling pathway were conducted by real time PCR analyses.

Results: We found that the NBF-SE had an antiproliferative effect on HT29 cells. In addition, NBF-SE induced sub-G1 cell cycle cessation and an increment in cell apoptosis, all in a dose- and time-dependent manner. Further, NBF-SE significantly up-regulated Caspase10 and Caspase3 genes, correlated to increased apoptosis. However, Kelch-like ECH-associated protein 1 (KEAP1) and Nuclear factor erythroid 2–related factor 2 (Nrf2) as well as Glutamate-cysteine ligase modulator (GCLM) - mediated antioxidation and anti-apoptosis pathway genes were upregulated, which was in contradiction with apoptosis activation.

Conclusions: We revealed NTBF as a new inducer of cell death confirmed by cytotoxic activity, cell cycle arrest and, increased apoptosis together with NTBF's greater potency in the activation of caspase apoptotic signaling pathway over NRF2/ GCLM antioxidant response. Moreover, NTBF strain ATCC-23745 may be a hidden probiotic and be used for further studies in CRC treatment.

Keywords: non-toxigenic *Bacteroides fragilis; Colorectal cancer (CRC), apoptosis; cell cycle;* Nrf2 Pathway

1. Introduction

The incidence of Colorectal cancer (CRC) ranks third among the most common cancers worldwide and the gut microbiome plays a vital role in its commencement and development. The microbiome is involved in CRC by modulating different cell signaling pathways or by producing metabolites causing tumor progression or suppression (M. Li *et al.*, 2021; Rebersek, 2021). One major gut microbiota species in the human colon is *Bacteroides fragilis*. Based on the existence of *bft* gene which is located on a pathogenicity island and encoded enterotoxin (BFT) or fragilysin, *B. fragilis* strains may be divided into two groups. (I) enterotoxigenic *B. fragilis* (ETBF) strains, containing *bft* gene and, (II) nontoxigenic *B. fragilis* (NTBF) strains, lacking bft gene (Claros *et al.*, 2006).

Still, *B. fragilis* plays an intricate role in a way that enterotoxigenic *B. fragilis*-derived toxins induced Diarrhea, inflammatory bowel disease (IBD) and CRC, while non-toxigenic *B.fragilis* (NTBF) promoted mucosal immune development or suppress colitis and inflammation-associated CRC (Chan *et al.*, 2019; Ryu *et al.*, 2020). NTBF also has a beneficial relationship with the host through modulation of the host's immune response by secreting outer membrane vesicles (OMVs) containing polysaccharide A (PSA). For instance, the T helper cells stimulated by PSA produce interleukin-10 (IL-10), which can prevent IBD or other inflammatory responses (Lee *et al.*, 2018; Wexler, 2007). Besides, some *Bacteroides* spp. like *Bacteroides thetaiotaomicron* (BT) ferment comestible fibers into some short-chain fatty acids (SCFAs) like propionate that repressed several histone methyltransferases which were presumed to have a functional role in CRC growth and metastasis (Okugawa *et al.*, 2015; Ryu *et al.*, 2020). SCFAs can also suppress the expression of proinflammatory cytokines and decrease colonic inflammation in colitis-associated colorectal cancer model (Tian *et al.*, 2018) and offers some protection against *Salmonella* (Jacobson *et al.*, 2018).

B. fragilis extract has a broad spectrum of molecules such as lipopolysaccharide (LPS) also known as endotoxin, amyloids, lipoprotein (LP), and non-coding

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** **List of abbreviations** :CRC: colorectal cancer; NBF-SE: B. fragilis sonicated extract; nontoxigenic B. fragilis (NTBF); KEAP1: Kelch-like ECH-associated protein 1; Nrf2: Nrf-(erythroid-derived 2)-like 2; GCLM: Glutamate-cysteine ligase modulator; LPS: lipopolysaccharide; OMVs: outer membrane vesicles; Casp10: Caspase-10; casp3: caspase-3; PSA: polysaccharide A; AREs: antioxidant response elements; ROS: reactive oxygen species; DISC: death-inducing signaling complex

RNAs (Lukiw *et al.*, 2019; Zamani *et al.*, 2020) that raise the generation of reactive oxygen species (ROS) (Ko *et al.*, 2020; Zhao *et al.*, 2014), leading to oxidative stress-induced cell damage. Thereby, multiple antioxidant defense systems may be activated. For instance, ROS overproduction oxidizes cysteines within KEAP1 which causes segregation of KEAP1 from NRF2. Hence, NRF2 activates without its suppressor (KEAP1) and interacts with antioxidant response elements (AREs) in the regulatory regions of over 200 genes to mediate cellular antioxidant responses (Liu *et al.*, 2021; Schieber *et al.*, 2014). Moreover, antioxidant pathways associated with NRF2 also increase the synthesis of endogenous antioxidant glutathione (GSH). GSH synthesis requires Glutamate Cysteine Ligase (GCL) enzyme which is comprised of 2 sub-units defined as catalytic (GCLC) and modifier (GCLM) subunits. The promoters for both GCLC and GCLM consist of AREs (Bea *et al.*, 2003; Saha *et al.*, 2020). Also, there is evidence that ROS overproduction disrupts mitochondrial membrane integrity, accompanied by the dislodge of cytochrome c from mitochondria into the cytoplasm to recruit cell initiator caspases. Caspase-10 is a key initiator caspase that cleaves and activates caspases 3 and 7. Over-activation of some caspases such as caspase-3 can lead to abrupt cell death (Bell *et al.*, 2017).

Therefore, we discussed the effects of non-toxigenic *B. fragilis* sonicated extract on cell viability, cell cycle and apoptosis of HT-29 cells in addition with the underlying Keap1/NRF2/Caspase3/caspase10/GCLM molecular pathway.

2. Materials and Methods

2.1. Bacterial culture and preparation of sonication-assisted extraction

NTBF *B. fragilis* (ATCC 23745) was obtained in a freeze-dried form (Pasteur Inst., Iran). The culture was performed in an anaerobe chamber. 1.0 mL of brain heart infusion (BHI) broth (Merck) was poured on a lyophilized bacterial pellet to rehydrate the entire content, then the aliquot was transferred into the tube containing 9 mL of BHI broth. Several drops of the suspension were inoculated on BHI agar, to check for purity. The cultures were placed in the anaerobe chamber at 37°C. At the exponential phase, bacterial cells were centrifuged. The bacterial pellet was washed and re-suspended with PBS to OD_{600} = 1.0 (equivalent to approximately 10^9 CFU/ml). Other needed concentrations were also adjusted using PBS. 10^9 CFU per ml concentration of *B. fragilis* was lysed using an ultrasonic homogenizer (Hielscher) and then was directly sterilized by a 0.22-µm filter membrane (Bioyfil). Filtrates were stocked in aliquots at -80 °C until required (Li *et al.*, 2017).

2.2. Cell culture

HT-29 human colorectal adenocarcinoma cells (National Cell Bank, Iran) were cultured in RPMI 1640 (Bioidea) with 1% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (Bioidea) in a $CO2$ incubator (HEPA, Thermo Fisher Scientific, Inc).

2.3. MTT cytotoxicity assay

The toxicity effect of *B. fragilis* sonicated extract (NBF-SE) was analyzed by using MTT assay (Sigma-Aldrich, Merck). Briefly, the 5×10^4 HT-29 cells/well were cultured in 96-well plates overnight and then treated with 10% (10^8 CFU/ml) and 100% (10^9 CFU/ml) concentrations of NBF-SE for 24 and 72 h. Negative controls were treated with equal volumes of BHI containing RPMI1640 medium. After this time of incubation, cells were further incubated with MTT solution (Merck, Germany) for one hour. Thereafter, DMSO was added and the plates were shaken for 20 minutes on a shaker. The absorbance of well plates was read by microplate reader (BIO–RAD) at 570 nm. All the experiments were replicated three times. Below is the formula used to calculate the survival percentage:

$$\% \text{ Cell viability} = (\text{Mean OD}_{\text{Sample}} - \text{Mean OD}_{\text{blank}}) / (\text{Mean OD}_{\text{Negative control}} - \text{Mean OD}_{\text{blank}}) \times 100.$$

2.4. Quantitative Real-Time PCR

After treatment of HT-29 cells with 10^9 CFU/ml NBF-SE for 24h, total RNA was isolated using the Cinnagen RNX extraction kit (Iran). RNA concentration and purity were assessed by measuring the absorbance at 260/280 nm with the Spectrophotometer (BioTeck). The cDNA was synthesized utilizing the easy cDNA synthesis kit (Parstous, Iran) following the manufacturer's guideline. qRT-PCR was performed using RealQ Plus 2x Master Mix Green kit (Ampliqon). The reactions were carried out in MyGo Pro real-time PCR Thermocycler (IT-IS Life Science). Primers were designed using IDT, and Primer 3 online software and were synthesized by Sinaclon. The primer sequences are reported in Table 1.

Table 1. Sequence of used primer pairs in qPCR.

		Primer sequence (5'->3')
KEAP1	Forward	AGACGTGGACTTTCGTAGCC
	Reverse	CCAGGAACGTGTGACCATCA
NRF-2	Forward	AGCCCCTGTTGATTTAGACGG
	Reverse	TGTCAGTTTGGCTTCTGGACT
GCLM	Forward	ACAGCCTTACTGGGAGGAATTA
	Reverse	ACCTGTGCCCACTGATACA
CASP 3	Forward	TGGAGGCCGACTTCTTGTATG
	Reverse	GCACAAAGCGACTGGATGAAC
CASP 10	Forward	CCGACAAAGGGTTTCTCTGT
	Reverse	TTGGGAAGCGAGTCTTTCAG
Gapdh	Forward	GTGGTCTCCTCTGACTTCAAC
	Reverse	GGAAATGAGCTTGACAAAGTGG
Hprt1	Forward	AAGGGTGTTTATTCCTCATGGAC
	Reverse	AGCACACAGAGGGCTACAA

The qPCR data was analyzed by Livak method ($2^{-\Delta\Delta CT}$) (Livak *et al.*, 2001) and normalized by the expression levels of housekeeping gene, GAPDH and HPRT.

2.5. Apoptosis analyses

In order to confirm whether the cytotoxicity induced by NBF-SE is indeed due to apoptosis,

Annexin-V Apoptosis Detection kit (BioLegend) was used. Briefly, HT29 Cells were seeded in 6-well plates and

incubated with 10^8 and 10^9 CFU/ml of NBF-SE for 24 and 72 h. The RPMI media containing BHI was used as a negative control. Following treatment, the cells were incubated with Annexin V/ propidium iodide (5 µl each) for 15 min under room temperature. Finally, the apoptotic cells were detected by a flow cytometer (BD FACSCalibur) and analyzed by FLOWJO software (USA).

2.6. Apoptotic index (AI) percentage

AI was measured by dividing the % apoptotic cells ($AnnexinV^+$) over the % total cells ($AnnexinV^+$ plus $AnnexinV^-$) using the following formula: $\%AnnexinV^+$ cells (apoptotic cells) / ($\%AnnexinV^+$ cells + $\%AnnexinV^-$ (Q1)) (Prieto *et al.*, 2002).

2.7. Cell Cycle Analysis

HT-29 cells were plated on 6-well plates. The following day, cells were treated with 10^8 CFU/ml of NBF-SE for 24 and 72 h. The RPMI media containing BHI was used as a negative control. After the specified time, cells were trypsinized, then harvested and rinsed with PBS and finally fixed in 70% ethanol. The fixed cells were suspended in staining solution containing PI/RNase A (50 µg/mL of each) for 20 min. The relative proportions of cells distributed in each cycle regions were calculated by flow cytometer (BD Biosciences).

2.8. Statistical analysis

Scientific software, Graphpad Prism 7.0, was used for analysis, graphing and statistical evaluation. Significant differences among multiple groups were determined using an unpaired Student's t-test, one-way analysis of variance (ANOVA) and, tukey's multiple comparisons test evaluate the main effect of NBF-SE on HT-29 cells. Data were reported as the mean ± standard error of the mean (SEM) and were considered statistically significant when the P value was *p<0.05, **p < .01, and ***p < .001 and, ****p<.0001 and indicated in the figures.

3. Results

3.1. Declines in HT-29 cell viability following NBF-SE exposure

With the intention of investigating the cytotoxicity of the obtained NBF-SE, the MTT assay was performed in two different concentrations (10^8 and 10^9 CFU/ml) and times (24 and 72h). As shown in figure 1, cell survival of HT-29 cells treated with NBF-SE was significantly decreased in comparison with the control group in a time and dose-related manner compared to the control group. The inhibitory concentration (IC50) of NBF-SE was ~10^9 CFU/ml after 24 h.

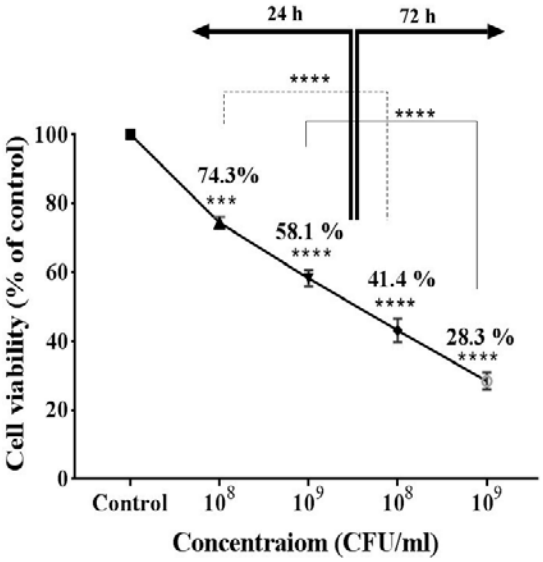

Figure 1. HT29 cells were treated with two different concentration of NBF-SE (10^8 and 10^9 CFU/ml) for 24 and 72h, then the cytotoxicity effect was measured by MTT assay. NBF-SE decreased the cell survival in a dose- time dependent manner. The results are reported as survival percentages compared with the control (p<0.05. *p < .05, **p < .01, ***p < .001 and, ****p<.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 NBF-SE

To examine whether NBF-SE has an apoptotic effect on HT29 over time and dose, annexin V/PI double staining was performed.

As shown by Fig 2A-F, NBF-SE clearly induced early and late apoptosis from a dose and time-dependent aspect compared with the control groups. Additionally, in both cases, increasing the concentration of NBF-SE from 10^8 to 10^9 CFU/ml and increasing the duration of exposure from 24 h to 72 h, has raised the percentage of the apoptotic index (AI) (Fig 2G, H).

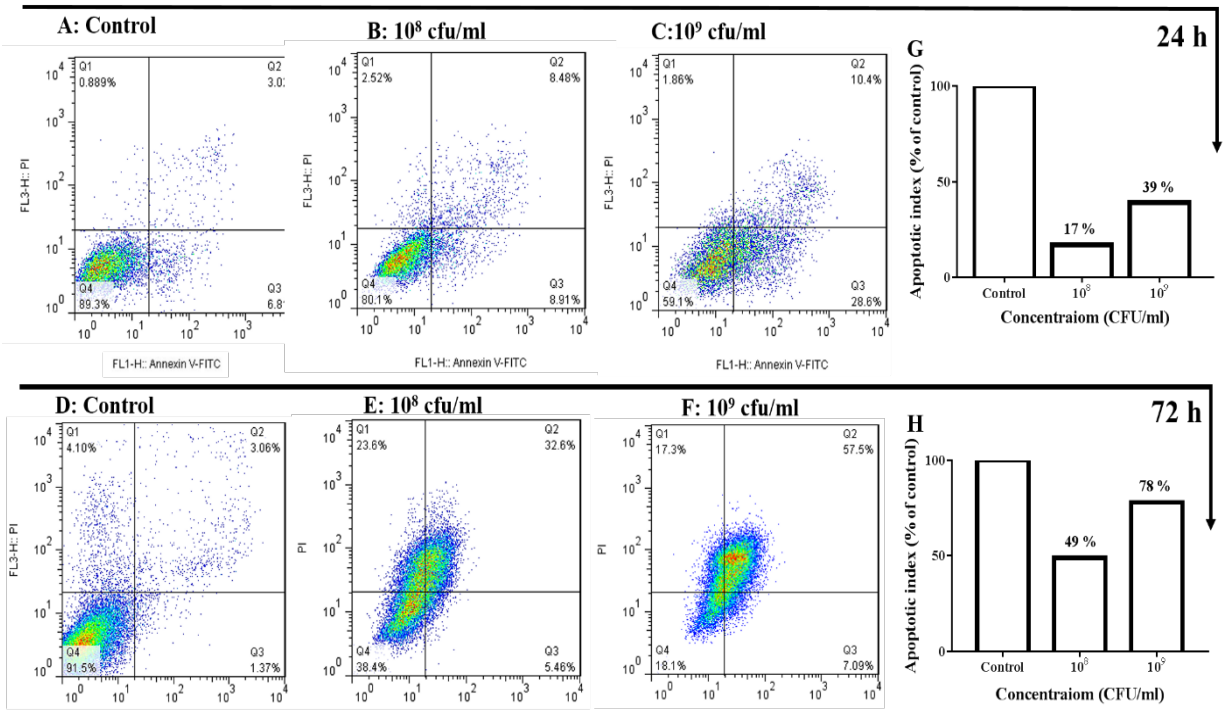

Figure 2. NBF-SE led to a significant increase in the percentage of apoptotic cells from a concentration and duration-dependent aspect. (A-F) Flow cytometry analysis was carried out after HT29 cells were treated with 10^8 and 10^9 CFU/ml NBF-SE for 24 and 72 h. The quadrants are as follows: Lower left belongs to viable cells, upper left shows necrotic cells, and lower right and upper right belongs to early and late apoptosis respectively. Apoptotic index (%) of HT-29 cells after 24 and 72, respectively (G, H).

3.3. NBF-SE induces subG1/G1 cell cycle arrest

Since cell cycle progression is one of the effective mechanisms in cell proliferation, any ingredient that arrests the cell cycle can be considered a possible candidate for an anti-cancer substance.

So, to better clarify the NBF-SE inhibitory effect, HT-29 cells were subjected to 10^8 CFU/ml NBF-SE for 24 and 72 h. Then cell subpopulations in sub-G1, G1, S and G2 phases were defined by flow cytometry. Percentage of cells in each phases of cell cycle showed a time-dependent Sub-G1 arrest. During 24h, NBF-SE treated cells showed a higher SubG1- G1 cell population (2.8%) compared to the control (subG1:0.9%), even though it was not statistically significant (Fig. 3). A concomitant reduction in S and G2 phase after 24h was also observed. Meanwhile, the extent of changes at 72h was more evident so that the ratio of treated cells in the sub-G1 phase (apoptotic cell population) was significantly increased from 2.8% to 42.2%. The significant observable decreases in cells at the G, S and, G2 illustrated no cell cycle arrest in those phases. This experiment suggested that NBF-SE induces growth arrest in subG1-phase.

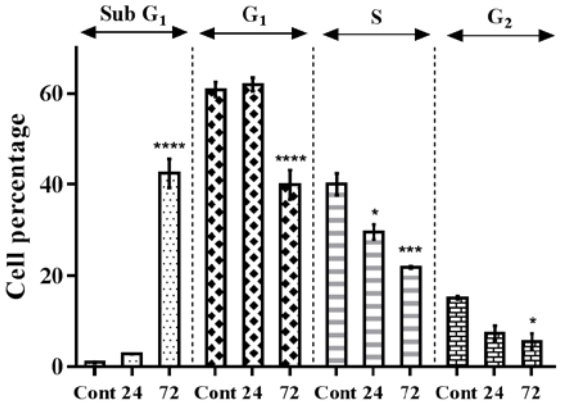

Figure 3. NBF-SE induces subG1-phase cell cycle arrest in HT-29 cells. Cells were treated with 10^8 CFU/ml NBF-SE for 24 h and 72 h, then the percentage of cells in each sub-G1, G1, S, and G2 phases of the cell cycle was calculated. Control groups are indicated as (Cont) and treated cells are indicated as 24 (24 h treatment) and 72 (72 h treatment). Each of the phases of the cell cycle is marked with an arrow at the top of the corresponding columns. Each data point represents the mean (±SEM) of three independent experiments (P<0.05: *, P<0.01: **, P<0.001: *** and, p<0001: ****).

3.4. Effect of NBF-SE on the expression of apoptosis-related genes

It was noticed in our previous test that the NBF-SE led to the subG1-phase cessation, which was conducted towards apoptotic cell death. In order to discover the cellular pathway which led to apoptosis, we investigated the NBF-SE effect on the expression of a hypothetical path consisting of Keap1/NRF2/ GCLM/Casp10/Casp3 genes. The relative expression of genes was measured by $2^{-\Delta\Delta Ct}$ method using GAPDH (Figure 4A) and HPRT (Figure 4B) housekeeping genes as normalizers. However, due to the lower variation in GAPDH expression (lower Δct values) compared to HPRT, GAPDH was chosen to report the results.

As shown in figure 4, significant over expression of 5 mediated apoptotic genes was observed after HT-29 cells were incubated with NBF-SE (10^8 CFU/ml) for 24 h. These results indicate that NBF-SE stimulated the transcriptional induction of NRF2 and its antioxidant response gene, GCLM. Additionally, increased expression levels of *initiator caspase-10 and* executioner caspases-3 were observed.

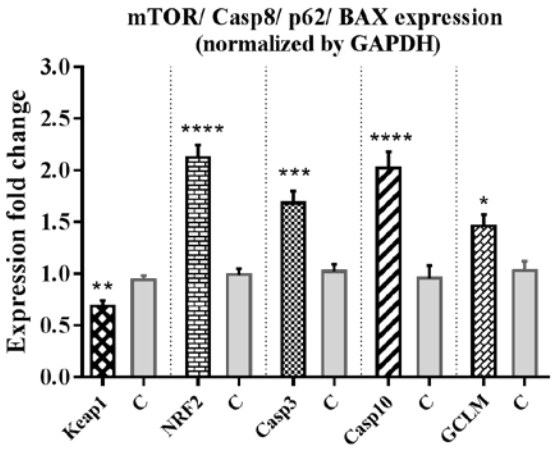

Figure 4. NBF-SE effect on the expression of hypothetical path consisting of the Keap1/NRF2/ GCLM/Casp10/Casp3-mediated apoptotic genes. HT-29 cells were exposed to 10^8 CFU/ml NBF-SE for 24 h. The relative expression of target genes was computed using A) GAPDH and B) HPRT as normalizers. The expression changes compared with control (denoted as 'C') are mentioned in diagrams as fold change. Data are represented as means (± SEM) of three independent experiments (P<0.05: *, P<0.01: **, P<0.001: *** and p<0001: ****). HT-29 cells treated with NBF-SE didn't show a significant change in Keap1 expression (*P*>0.05).

4. Discussion

Commensal gut microbiota actuates the immune system of the host, resulting in protective responses against pathogens (Bae *et al.*, 2022), supplied the host with essential nutrients, metabolize indigestible carbohydrates, and produce certain nutrients such as short-chain fatty acids (Sánchez-Alcoholado *et al.*, 2020). *B. fragilis* can be considered a friendly commensal. The capsular Polysaccharide A (PSA) of *B. fragilis* is capable of stimulating T helper cells to produce anti-inflammatory cytokine IL-10, which is essential to mediate the generation of a normal mature immune system and preventing abscess formation (Chang *et al.*, 2017; Lee *et al.*, 2018; Wexler, 2007). It has been shown that the treatment of CRC cells with purified PSA from *B. fragilis* inhibited the proliferation of CRC cells via the production of the IL-8 pro-inflammatory cytokine and suppressed cell migration and progression of the cell cycle (Sittipo *et al.*, 2018). Furthermore, most approved probiotics based on intestinal microbiota belong to lactic acid bacteria (LAB), but new species and genera are being assessed for future use. Recently, a new strain of *B.* non-toxigenic *fragilis* named ZY-312 is shown to exert beneficial probiotic effects and was recommended as the first probiotic candidate from the phylum Bacteroidetes (Deng *et al.*, 2016; Wang *et al.*, 2017).

Therefore, we believe non-toxigenic *B. fragilis* strain ATCC-23745 can be a fine choice to explore its capability as probiotic bacteria on growth inhibition, apoptosis induction, and blocking the cell cycle. Hence, in the present study, we assessed the impact of non-toxigenic *B. fragilis* strain ATCC-23745 sonicated extract (NBF-SE) on the colorectal adenocarcinoma HT29 cells. Our findings revealed a significant reduction of viable cells after co-incubation of HT29 cells with NBF-SE over time- and dose-dependent manner. Similarly, NBF-SE was associated in triggering early and late apoptosis of HT-29 cells depending over the time and dose of exposure, also could raise apoptotic index (AI) about 1.5-2-fold more than the control. This was also confirmed using qRT-PCR analysis. We observed greater expression of caspase-10 and caspase-3 genes, which indicated the caspase-dependent apoptosis following NBF-SE treatment.

In a relevant work using nontoxigenic *B. fragilis* ATCC 23745 strain (Miranda *et al.*, 2008) similar to that of our study, the phenylacetic acid (PA) of the sonicated extract could both induced vacuolating effect on Vero cells and peritoneal macrophages and increased apoptotic cell death (Falcão *et al.*, 2015).

Since excessive proliferation is a well-recognized hallmark of human cancer, therefore any agent that can arrest cancer cell cycles may be an efficient anticancer substance. By using flow cytometry analyses, we noted a time-dependent inhibitory effect of NBF-SE on HT-29 cells which was reliant on the cell cycle (sub-G1 phase) arrest. The percentage of sub-G1 cells increased significantly (42.2%) after 72 h, while the percentage of cells decreased about 2-3 fold at S and G2 phases after both 24 and 72 h.

Genetic research on *B. fragilis* suggested the downregulation of cell cycle-activating genes in HT29 cells treated with e those effective concentrations of *B. fragilis* PSA (Sittipo *et al.*, 2018).

However, there is something that contradicts the probiotic bacteria property in favor of human health. LPS, the major component of non-toxigenic *B. fragilis* outer membrane, is capable of stimulating apoptosis-inducing receptors. These transmembrane receptors then bind to other proteins to stablish DISC assembly and ROS overproduction. Upon the formation of DISC, apical initiator caspase 8 activates effector caspase 3, leading to apoptosis (Wachmann *et al.*, 2010). Also, these byproducts result in antioxidant pathways induced by NRF2 for maintaining the stability of cell's internal environment. There was also a report that LPS increased the expression of proapoptotic factor (BAX) and Nrf2 in wild-type (WT)

mice. Nrf2 then separates from its inhibitor, Keap1 and targets its downstream antioxidant response elements (AREs) such as GCLC and GCLM (two subunits of glutamate cysteine ligase) (Y. Li *et al.*, 2021).

Therefore, we detected the levels of Keap1/NRF2/Caspase3/caspase10/GCLM to estimate the intracellular homeostasis state. Significant upregulation of Nrf2 and GCLM (Nrf2 target gene) and downregulation of Keap1 (Nrf2 inhibitor), reveals the activation of the antioxidant and anti-apoptosis pathway. The implementation of this protective path was against NBF-SE -induced oxidative damage. However, by looking at the results of casp10 and casp3 overexpression, increased cell apoptosis and, greater cell populations in sub-G1 phase (apoptotic cells), we speculate that the induction of Nrf2/GCLM expression was blunted by NBF-SE. This may be due to greater potency of caspase apoptotic signaling over NRF2/GCLM antioxidant pathway (Fig 5).

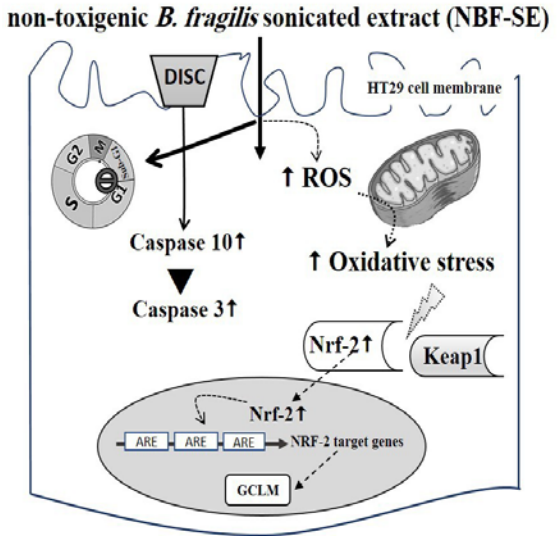

Figure 5. Possible mechanism of NBF-SE- induced apoptosis in HT29 cells. Stimulation of extrinsic death receptor-dependent pathways triggers the activation of the death-inducing signaling complex (DISC). This complex may then recruit a caspase-dependent cell execution pathway via the caspase-10 caspase-3 route, leading directly to apoptosis. Furthermore, during the stressed conditions, the amount of cellular NRF2 increased upon exposure to oxidative stress, ROS overproduction and oncogenic signaling. ROS reaction with sensor cysteines of KEAP1 protein disrupts the KEAP1-NRF2 complex. Nrf2 then translocates into the nucleus and targets its downstream antioxidant response elements (AREs) such as GCLC and GCLM (two subunits of glutamate cysteine ligase) to address oxidant stress. Dotted lines (- - -) indicate the weak path and continuous lines (—) indicate the main dominant pathway.

To clarify the validity, it is suggested to re-measure the apoptosis level and expression of caspases after increasing the expression of Nrf2 mRNA in cells by the Nrf2 expression vector. We also suggest 1- finding more involved apoptosis signaling pathways correlated to CRC, 2- scanning of effective apoptotic substances in NBF-SE and 3- examining additional cancer and normal cell lines along with untried recent microbiota.

5. Conclusions

In conclusion, we have demonstrated that NBF-SE eventuates in an antiproliferative and apoptotic effect on human CRC cell line along with arresting the cell cycle progression in the subG$_1$ phase dependent over the time and dose of exposure. We have also uncovered that NBF-SE promoted apoptosis through the Casp10/Casp3 signaling pathway while making the antiapoptotic Keap1/NRF2/ GCLM path less effective.

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