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Nano-Exopolysaccharide from Probiotic *Lactobacillus brevis* Impact against Cancer Cells Caco and HT-29 Development based on ROS, IL-6, IL-8, TNF-β and M30

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ABSTRACT

Probiotic developments recently have provided new avenues for the fight against cancer. Via making use of *Lactobacillus brevis* extract's reducing properties. Therefore, the current study investigated whether nano-exopolysaccharide (NEPS) from probiotic can decrease cell proliferation, increase apoptosis, and activate autophagy in CaCo2 and HT-29 cells using *in vitro* techniques. On the other hand, compared to non-apoptotic cells, apoptotic cells showed orange-colored bodies as a result of cellular shrinkage and hemorrhage brought on by NEPS stimulation. Not to be overlooked is the NEPS 's exceptional selectivity in causing cell death, particularly in CaCo2 and HT-29 cells. For CaCo2 and HT-29 cells, the half-maximum inhibitory concentration (IC50) values were 9.4 µg/ml and 6.2 µg/ml, respectively. In HT-29 cells, probiotics cause an increase in the synthesis of cytokines linked to inflammation, such as TNF-β, IL-8, and IL-6. As an outcome of apoptosis being triggered and immune system responses being strengthened, our study's findings suggest that using probiotics could be a practical approach to eliminating colorectal malignant cells.

INTRODUCTION

Nowadays, probiotics are generally accepted as helpful bacteria that benefit humans in a variety of ways; the most popular ones are *Lactobacillus* and *Bifidobacterium* (Chong, 2014; Ebel *et al*., 2014; Iqbal *et al*., 2014; Ho *et al*., 2014; El-Waseif *et al*., 2021a). Probiotics have the potential to be employed in therapeutic settings for a variety of conditions, including diabetes, oral health, atopic illnesses, intestinal diseases, urogenital infections, viral infections, and potentially cancer (Uccello *et al*., 2012; Gou *et al*., 2014; Abd-Elwahed *et al*., 2023; Qadah *et al*., 2023). To date, a number of investigations have been carried out to clarify the relationship between probiotics and colorectal cancer. Probiotics have the power to reduce oxidative stress in the gut and preserve the equilibrium of microflora. In fact, some research has suggested that taking probiotics may help prevent cancer (Kahouli *et al*., 2013; El-Waseif *et al*., 2021b; El-Waseif *et al*., 2022; Hegazy *et al*., 2023).

One of the most common and fatal types of cancer in the world is colorectal cancer (CRC). The initial phase of protocol therapy for CRC patients involves resection surgery, followed by chemotherapy (e.g., leucovorin and 5-fluorouracil in combination with oxaliplatin or irinotecan), and target therapy using peptides or monoclonal antibodies that target EGFR or VEGF(Van Cutsem *et al*., 2010; Van Cutsem *et al*., 2014; Vallino *et al*., 2023). Unfortunately, scattered metastases present at diagnosis limit the efficiency of modern anticancer treatments (Longley *et al*., 2006; Arnold *et al*., 2017). Evidence from experiments and clinical settings highlights the part played by the inflammatory tumor microenvironment in the onset and progression of colorectal cancer (Wang and Karin, 2015). In this case, the primary offender is thought to be inteurleukin-6 (IL-6), which is secreted by cancer cells and stromal cells, namely cancer-associated fibroblasts. It has been demonstrated (Foran *et al*., 2010).

Serum levels of IL-6 were consistently reported to be significantly greater in CRC patients compared to healthy controls. These levels were linked to metastases, accelerated tumor development and aggressiveness, and poor clinical outcomes (Knüpfer and Preiss, 2010; Waldner *et al*., 2012; Toyoshima *et al*., 2019). It has been demonstrated in this context that the intestinal inflammatory condition and the microbiota have a reciprocal influence that ultimately affects the development and progression of colorectal cancer (Privitera *et al*., 2022).

By improving the integrity of the intestinal epithelial barrier, increasing the accumulation of antioxidants and anti-carcinogenic metabolites against CRC, reducing intestinal inflammation state (e.g., IL-6, IL-8, TNF-β), and protecting against carcinogenesis, probiotics and their metabolites have a positive, both qualitatively and quantitatively, impact on the composition of the microbiota in the intestinal tract and also control metabolic activity (Molska and Reguła 2019; Guiomar *et al*., 2022; Zeng *et al*., 2022).

In the present study, we attempted to observe the efficacy of taking personalized nano-exopolysaccharide on cytotoxicity activity of treated CaCo2 and HT-29 cells based on the ratio reactive oxygen species (ROS), inteurleukin-6 (IL-6), IL-8 and tumor necrosis factor (TNF-β). Also, the amount of M30 that was present in cell extracts.

MATERIALS AND METHODS

Probiotic Culture:

Lactobacillus brevis strain was kept in an MRS medium containing 50% glycerol at -80 °C, (De Man *et al*., 1960). According to the 0.5 McFarland standards, the turbidity of the bacterial growth was evaluated after 48 hours of static incubation at 30°C with an ordinary inoculum of 100 microliters ($1x10^7$ cells/ml) of bacterial cell culture. The nano-exoPolysaccharide (NEPS) was synthesized and characterized in pervious study.

Cytotoxicity Assay:

The 3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, USA) assay was slightly modified to determine the cytotoxic profile of sample at concentrations (250, 500, 1000 and 1250) µg/ml, which were dissolved in propylene glycol (Sigma-Aldrich, USA) and diluted before use using a culture medium to the indicated concentration 0.1% (v/v) . The control set consisted of cells of CaCo2 and HT-29 that had only received the vehicle treatment. In a tissue culture plate (96-well), the wells were inoculated with $1 X 10^5$ cells/ml (100 µl/well) and then incubated for 24 hours at 37 ºC to produce a complete monolayer sheet. Decanting the growth medium and twice washing the cell monolayer with washing media came after the creation of a confluent cell sheet. Subsequently, the sample underwent two dilutions in RPMI medium supplemented

with 2% maintenance serum medium. After that, three control wells received simply maintenance medium, and a volume of 0.1 ml of each dilution was tested in distinct wells (Van *et al*., 1994).

After incubation, the plate was inspected. Physical indicators of toxicity, such as shrinkage, rounding, or loss of the monolayer wholly or partially, were examined in the cells. A 5 mg/ml MTT solution in PBS was made, and 20 µl of the solution was added to each well. Next, placed on a platform that shakes (150 rpm for five minutes) to thoroughly mix the MTT into medium. MTT metabolization is enabled by an incubation period of 1-4 hours at 37 ºC and 5% CO_2. After removing the media, formazan (MTT metabolic product) crystals needed to dissolve, thus it was thoroughly mixed in 200 µl DMSO. An enzyme-linked immunosorbent assay (ELISA) plate reader (BioTeck, Bad Friedrichshall, Germany) was then used to assess the absorbance at 570 nm in relation to cellular density.

% viability --(AT-Ab/ Ac-Ab) X 100

At.. Absorbance of (cells and probiotic conc.)

Ab.. Absorbance of media only

Ac.. Absorbance of negative control (cells and media only)

The half-maximum inhibitory concentration (IC50) was computed using GraphPad Prism 8.2.4.

Microscope and a digital camera, cell morphology was captured (Nikon, Japan). Every experiment was run in three triplicates

Determination of Apoptosis:

After receiving the nano-exopolysaccharide (NEPS) therapy, the CaCo-2 cells were collected and washed once with PBS (pH 7.2). After that, cells were stained with ethidium bromide (EB) (100 µl of 1 mg/ml) and acridine orange (AO) (50 µl of 1 mg/ml) on clean microscope cover slides. After being incubated for 20 minutes at 37 ºC in the dark, cells were collected and observed using a fluorescence microscope (ZOE fluorescent cell imager; BioRad, USA) (Abid-Essefi *et al*., 2023). Using an inverted.

Determination of Intracellular ROS Production:

Washing the cells, adding 1 ml of ROS assay buffer, 10 of 1 µ1x ROS assay dye solution, and gently mixing the wells completed the procedure. After that, the plate put in an incubator set to 37 ºC and 5% CO_2 for 60 minutes. Following the incubation period, the cells were treated with 50 µg/ml of SAG-Sh-SeNPs, and the production of ROS was promptly assessed using a fluorescent cell imager (ZOE BioRad, USA) with a fluorescent filter at 520 nm (González-Flores *et al*., 2014).

The M30 Apoptosense Assay:

The percentages of the probiotic strain were measured using an easily accessible ELISA kit, M30-Apoptosense (PEVIVA AB, Bromma, Sweden). As was previously mentioned (Kramer *et al*., 2004), apoptosis was also measured in CaCo-2 cells. The M30 Apoptosense test is used to quantify the soluble apoptosis-related and caspase-cleaved K18 (ccK18) fragments that include the K18Asp396 neo-epitope. This epitope is only accessible during K18's proteolytic processing by caspases 3/7/9. The method is a solid-phase sandwich enzyme immunoassay that uses an anti-K18 solid phase capture antibody, the HRP (horseradish peroxidase) conjugated M30 antibody, which is targeted against the K18Asp396 neo-epitope. Using a spectrophotometer to measure the absorbance at 450 nm, one can ascertain the analyte's concentration.

Quantification of Cytokines in Probiotic-Treated Cells Supernatants:

Nano-exopolysaccharide -treated HT-29 culture supernatants were used to measure the levels of TNF-β, IL-6, and IL-8. In 96-well plates, 10,000 HT-29 cells were seeded. Following a 24-hour adhesion period, the cells underwent a 3-hour treatment with a 50 µg/mL probiotic sample. The amount of cytokines in the culture supernatants was quantified using

ELISA. The average value ± standard deviation (S.D.) from a minimum of three duplicate tests is displayed as the result. The amount of cytokines in the culture supernatants was assessed using the sandwich enzyme-linked immunosorbent assay. Each cytokine was tested using a different ELISA kit (bioscience, San Diego, CA, USA), as directed by the manufacturer. The optical density of the reaction products was measured using an ELISA plate reader (EnSpire Multimode Plate Reader).

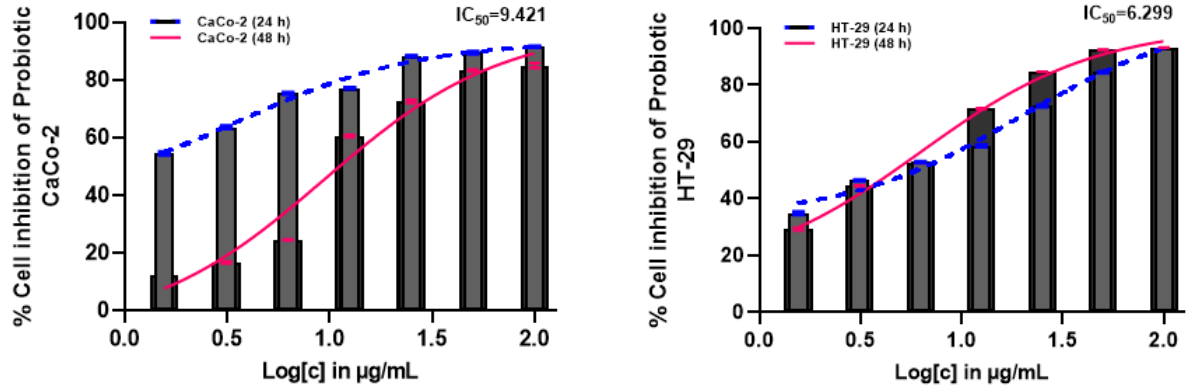
Statistical Analysis:

GraphPad Prism version 9.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze the data. Data were compared across numerous groups using an ANOVA, then between individual groups using Tukey's post-hoc test. For every test, the significance level was set at $P < 0.05$, and the least significance difference test was employed to ascertain variations in means.

RESULTS

Colorectal Anti-Cancer Impact Of Nano-Exopolysaccharide (NEPS) against CaCo2 and HT-29:

After being exposed to the probiotic for 24 hours, the viability of both cell lines decreased in a dose-dependent manner. Probiotics suppressed cell proliferation (IC50 9.4 µg/ml and 6.2 µg/ml, respectively), according to experiments using CaCo2 and HT-29 (Fig. 1). This suggests that after 48 hours, NEPS were more successful in slowing the development of CaCo2 cells than HT-29 cells. This might be because CaCo2 cells are more vulnerable to the effects of probiotics than HT-29 cells. Furthermore, the NEPS adherence to the cell membrane and disruption of the cell cycle may be the reason for the reduction in cell viability. The NEPS inhibited cell proliferation in both cell lines in a dose-dependent manner, with greater inhibitory efficacy in the CaCo2 and HT-29 cells, according to the MTT experiment. Furthermore, following a 48-hour treatment period, the inhibition was also noticeable in CaCo2 cells. This suggests that probiotics may influence CaCo2 and HT-29 cells in a sustained manner.



Fig; 1: Growth inhibition curves in CaCo2 cells treated with NEPS; HT-29 cells treated with NEPS.

Effect of NEPS on Apoptosis:

The NEPS therapy caused the cells to undergo apoptosis, which was visible when the cells were stained with AO/EB. Under a microscope, observations of the fluorescence of healthy control cells showed that an AO component had diffused into the cell membrane, giving the cells their green hue. In contrast to non-apoptotic cells, cells that have died were found to exhibit orange-colored bodies as a result of cell shrinkage and bleeding brought on by the NEPS treatment. Early-stage necrotic cell nuclei with deposits of yellow-green AO staining are characteristic of apoptotic cells. The EB staining on the nuclei of late-stage apoptotic cells grew more intense and asymmetrically located as the apoptotic

stage advanced. Necrotizing cells had a larger volume and an irregular, orange-red fluorescence around the periphery of the cell (Figs. 2-3).

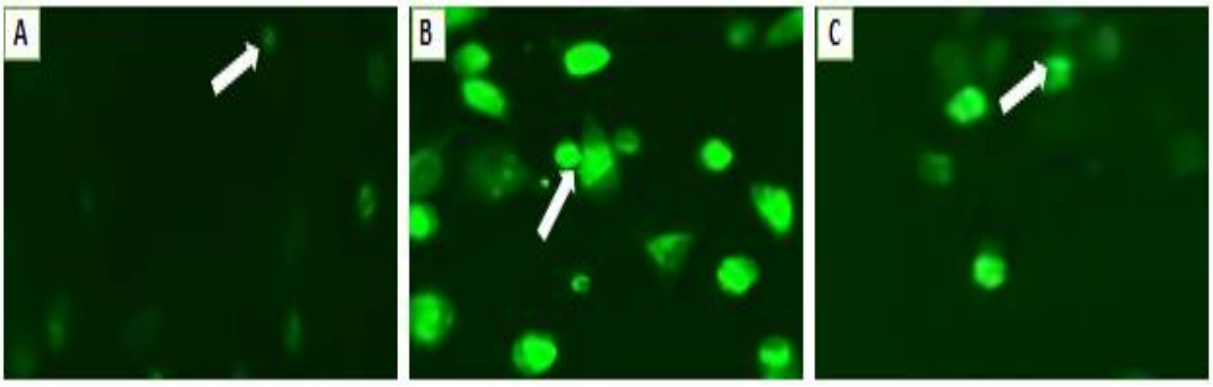


Fig. 2: The influence of NEPS on the production of reactive oxygen species (ROS) in CaCo-2 cells; (A) CaCo-2 cells in good health that are used as a control and do not glow green (there are no ROS);(B) CaCo-2 cells that have been treated with 20 µg/ml of NEPS and have green fluorescence (this indicates increase of ROS);(C) When cells were pre-treated with 5 mM GSH before being co-treated with 50 µg/ml of NEPS, there was a reduction in fluorescence.

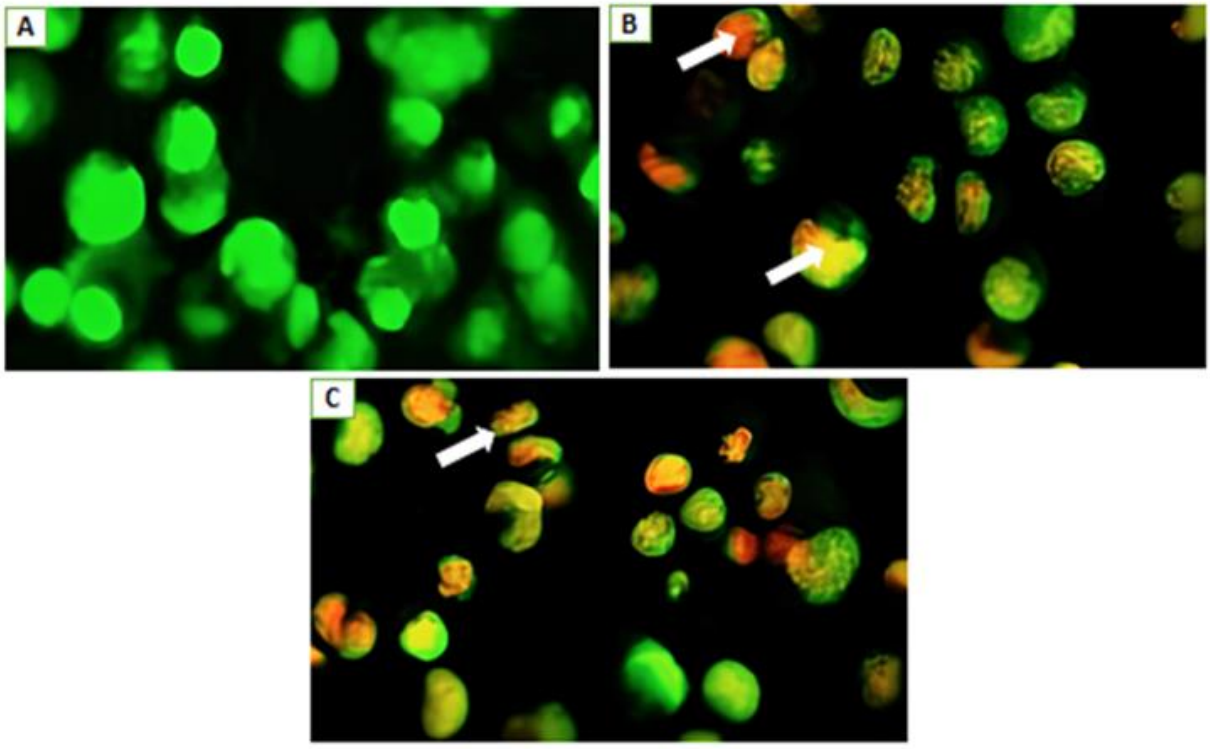


Fig. 3: (A) control and (B),(C) 50 µg/ml NEPS treated-Caco cells. [Arrows represent marked early-stage apoptotic cells (chromatin condensation) and late-stage apoptotic cells].

Induction of Apoptosis in CaCo2 Treated with NEPS:

The M30 epitope, a marker of caspases 3/7/9 activation and consequent death, was shown to be more concentrated in probiotic-treated cells than in the control group in CaCo2 cell extracts (Fig. 4). The highest amounts were seen 24–48 hours into the treatment. This suggests that probiotics can cause CaCo2 cells to

undergo apoptosis, possibly as a result of the oxidative stress they induce. Oxidative stress has the ability to activate caspases, which in turn causes the formation of M30 epitopes and apoptosis. Given that probiotics can cause cancer cells to undergo apoptosis, this suggests that they might be a useful therapeutic agent for treating some types of cancer. Additionally, NEPS can be used in combination with other therapies, such as chemotherapy, to further increase their efficacy.

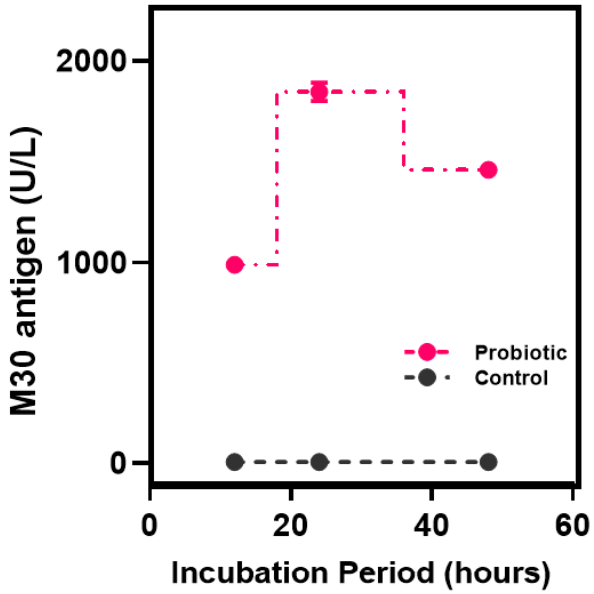


Fig. 4: The amount of M30 that was present in cell extracts was measured. Caco-2 cells were given a NEPS treatment of 50 µg/ml over a period of 24–48 h. Using the M30 Apoptosense ELISA kit, the amount of M30 that was present in cell lysates was determined.

Influence of NEPS -Treated HT-29 Cells on the Production of Pro-Inflammatory Cytokines:

Nano-exopolysaccharide cause an increase in the production of inflammation related cytokines, such as TNF- β IL-8 and IL-6, in HT-29 cells (Fig. 5). Immune response is enhanced by these cytokines, which are part of the body's natural response to infections and injuries.

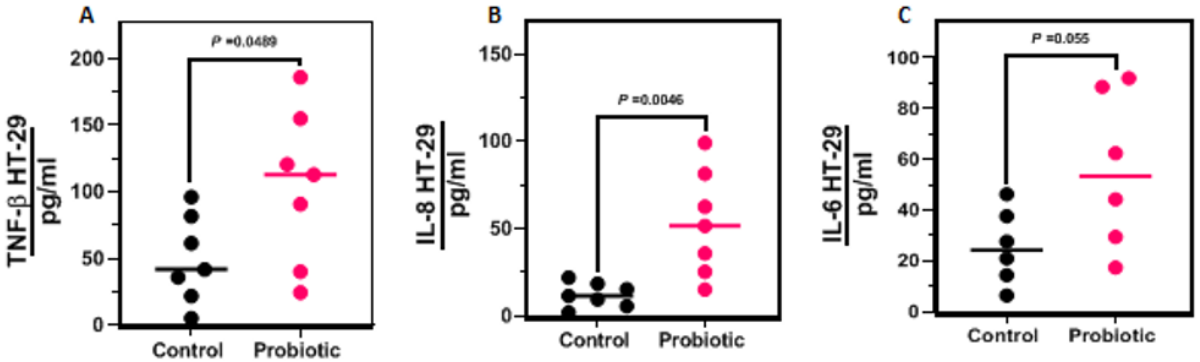


Fig. 5: The production of TNF-β, IL-8 and IL-6 by NEPS -treated HT-29 cells. A NEPS concentration of 50 µg/mL was added to the cells for a period of three hours. ELISA was used to determine the cytokine concentrations that were present in the culture supernatants. The findings are reported as the mean±SD ($P < 0.05$, Student's t-test) of three replicates**.**

DISCUSSION

In recent decades, a great deal of work has gone into developing effective anti-cancer medicines. NEPS are dependent on the biological behavior of the targeted cell. Thus, by inducing oxidative stress-mediated autophagy and/or apoptosis, they may improve conventional therapies. The current study has concentrated on the potential use of NEPS in tumor therapy as a means of creating new, efficient treatment instruments. Probiotics have been studied for their capacity to trigger apoptosis in colon cancer cells as well as their potential to influence the immune response to these cells in order to characterize feasible prospects for employing them to treat cancer. To do this, we looked at the signaling pathways involved in the apoptosis of colon cells.

Using AO/EB staining of the cells, our data showed that NEPS administration causes apoptosis in the cells. This might be explained by the fact that taking probiotics reduced the potential of the mitochondrial membrane, which in turn increased the production of reactive oxygen species (ROS), which in turn triggers the process of apoptosis in which cells die. Moreover, probiotic therapy increased the expression of apoptotic proteins such caspase-9 and Bax (Vallino *et al*., 2023). Furthermore, research (Vemuri *et al*., 2018) demonstrated that probiotic-induced excess ROS formation and ATP synthesis disruption impair the proper operation of the mitochondrial respiratory chain, leading to death of the mitochondrial pathway. Their investigations' outcomes offer compelling early evidence that biogenic probiotics, which trigger the apoptotic process in colon cancer cells, are effective against those cells *in vitro*. The existence of the bio molecular layer on the probiotic's surface, which is anticipated to be crucial to the apoptosis that probiotics elicit in colon cancer cells (Dong *et al*., 2012), as detailed below, supported these observations. The current investigation showed that the growth of CaCo2 and HT-29 cancer cells was suppressed by 50 μg/ml NEPS. Notably, probiotics induced intrinsic apoptotic pathways in colon cancer cells. The bio molecular layer of probiotics interacts with proteins on colon cancer cells to cause a series of events that can culminate in apoptosis. At these events, caspases, which are the main executors of apoptosis, become activated.

Additionally, by counting the number of living cells, the MTT assay can evaluate a substance's cytotoxicity in the context of cancer treatment. The MTT results showed that NEPS, at a concentration of 50 μg/ml, were hazardous to CaCo2 cells. Probiotics also demonstrated a strong potential to suppress cell proliferation, with the most notable suppression happening at 100 μg/ml. This suggests that NEPS might be used to cure cancer. Similar studies by other researchers have shown that probiotics cause cytotoxicity in a variety of cancer cell types, including as human cervical cancer, breast cancer, and oral cancer cell lines (Azad *et al*., 2018). This might be explained by the fact that probiotics disrupt cell membranes and induce oxidative stress in cells, both of which can result in cell death. Furthermore, probiotic can cause apoptosis, a programmed cell death process, in cancer cells and could potentially kill cancer cells, making them a potential cancer treatment (Ho *et al*., 2014).

In this work, we demonstrated that consuming probiotics that have the highest concentrations of TNF-β, IL-8, and IL-6 can increase the NK cells' cytotoxicity. This finding also raised the possibility that differing or opposing effects amongst strains of probiotics may be experienced by the hosts. Therefore, it should be mentioned that a thorough analysis of probiotics is necessary if we wish to get a specific result from them, since they may be beneficial to some individuals but ineffective to others. As previously indicated, some research has been done to demonstrate the benefits of

NEPS in increasing TNF-β, IL-8, and IL-6 function in mice and human models (Ho *et al*., 2014; Azad *et al*., 2018). Nevertheless, no thorough investigation has been done to look into the relationships between probiotics and TNF-β, IL-8, and IL-6 activity.

Numerous studies have revealed a strong correlation between the levels of TNF-β, IL-8, and IL-6 and NK activity. A long-term survival study of patients with gastrointestinal stromal tumors showed that increased production of NK cells and TNF-β was predictive of these patients' survival (Kramer *et al*., 2004). A different study revealed that individuals with colon cancer had lower levels of TNF-β and NK cytotoxicity, and that this decline was linked to the illness's advancement (Azad *et al*., 2018). TNF-β has been shown to increase the cytotoxicity of NK cells by making tumor cells more susceptible to the NK-mediated death process. As a matter of fact, dendritic cells (DCs) can create IL-12 in response to NK cells releasing IFN-γ, and IL-12 in turn will increase NK cells' production of IFN-γ cell. The innate immune response is made up of this feedback mechanism (Ho *et al*., 2014). In addition to IFN-γ, a study showed that NK cells released IL-10 to prevent DCs from producing IL-12 during systemic infections by viruses. Another study showed that NK activity was inhibited by IL-10 when there was a bacterial infection. Together, we suggested that the indirect impact is the mechanism by which probiotics cause NK cell-mediated cytotoxicity. For instance, highly active probiotics that produce IFN-γ can stimulate DC cells, causing DCs to secrete IL-12, which helps maintain a healthy DC to NK feedback loop (Ho *et al*., 2014).

Conclusion

This study demonstrates that in colorectal cancer CaCo2 and HT-29 cells, NEPS have an inhibitory effect on cell proliferation and cause apoptosis. The current study clarifies the NEPS ' possible apoptotic anti-colorectal properties. NEPS were found to have a cytotoxic effect on CaCo2 and HT-29 cells, preventing their proliferation, with IC50 values of 9.4 μg/ml and 6.2 μg/ml, respectively. NEPS therapy was found to significantly induce apoptotic cell death in colorectal cells, according to studies on apoptotic activity. After a 48-hour NEPS treatment that resulted in the release of pro-inflammatory cytokines, this impact was seen. The combined results of our studies offer additional proof of the possible anti-cancer effectiveness of NEPS as a workable substitute treatment approach for colorectal cancer. To validate these findings, however, more in vivo study employing animal models is necessary. NEPS have a unique structure and makeup that allows them to target tumor cells specifically. Additionally, they have the ability to connect to specific receptors on neoplastic cells, which increases the effectiveness of their induction of cell death. The probiotics also have immunostimulatory qualities, which aid in the further inhibition of tumor cells.

Declarations:

Ethical Approval: Not applicable.

Conflicts of Interest: The author declares no conflicts of interest.

Authors Contributions: All authors contributed towards the study design, experiment execution, data analysis, and manuscript drafting.

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Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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