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Effect of chitosan nanoparticles conjugated with the cell free supernatant of *Bifidobacterium bifidum* on the expression of genes related to colorectal cancer in colon adenocarcinoma (Caco-2) cell line

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Abstract

Background Colorectal cancer (CRC) is emerged as a global problem with high mortality rate; hence, finding of alternative treatment approaches is essential. The purposes of this research are to assess the impact of chitosan nanoparticles conjugated with the cell free supernatant of *Bifidobacterium bifidum* (CTNP/B.b-sup) on genes associated with CRC signaling pathways.

Methods The novel nano-drug were fabricated via an ionic gelation technique and analyzed using transmission electron microscopy (TEM) and dynamic light scattering (DLS) methods. The release of protein and entrapment efficiency (EE) of CTNP/B.b-sup were assessed using a BCA protein assay. Following an investigation into the toxicity of CTNP/B.b-sup on Caco-2 cells by MTT assay, the expression of genes associated with CRC signaling pathways was evaluated utilizing real-time PCR method.

Results CTNP/B.b-sup exhibited a suitable morphology with a particle size of 453.1 ± 230.8 nm and zeta potential of 9.11 ± 3.6 mV. The protein released was 75.5% at pH ~ 6.8 within 48 h with 83.3% of EE. The viability of Caco-2 cells against CTNP/B.b-sup was 90.3%. The effects of CTNP/B.b-sup on the expression levels of various oncogenes reveal a significant decrease in the expression of *β-Catenin*, *PI3K*, *TGF-α*, *Bcl2*, *TLR4*, *CEA*, and *TGF-β* oncogenes by 0.96, 0.37, 0.03, 0.41, 0.88, 0.69, and 0.71-fold, respectively. CTNP/B.b-sup induced the most significant reduction in *TGF-α* oncogene expression, with a decrease of 0.03-fold. Conversely, the strongest induction was observed in the expression of *Caspase9* suppressor, with a 73.4-fold increase.

Conclusion In the present study, the CTNP/B.b-sup was demonstrated to possess the capability of modulating genes associated with CRC progression, thereby highlighting its significant pro-apoptotic potential. It can be concluded that CTNP/B.b-sup is a suitable drug delivery system with anticancer properties, which can be regarded as a complementary therapeutic approach for the treatment of CRC.

Keywords *Bifidobacterium bifidum*, Chitosan nanoparticle, Colorectal cancer, Caco-2 cell line

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Introduction

Colorectal cancer (CRC) is emerged as one of the most common cancers globally, notable for its high fatality rates [1]. Conventional treatment modalities for CRC, encompassing endoscopy, surgery, chemotherapy, immunotherapy, and radiotherapy, frequently result in a multitude of adverse effects, thereby adversely impacting patient survival rates [2]. Consequently, there is an imperative to explore novel treatment approaches. In recent years, there has been a notable increase in research focusing on the potential of probiotics in cancer treatment [3].

Probiotics are defined as living microorganisms that have a positive impact on the gastrointestinal system. Probiotics have been shown to possess anti-inflammatory and tumor-suppressing properties [3]. A study reported that the cell-free supernatant (CFS) of bifidobacteria species significantly inhibited the development of colon cancer cells. The mechanism of action of the aforementioned species involves the down-regulation of anti-apoptotic genes and the up-regulation of pro-apoptotic genes, thereby inhibiting colorectal cancer [4]. According to the United States Food and Drug Administration (USFDA), probiotics such as *Bifidobacterium bifidum* (*B. bifidum*) are generally regarded as safe for human use and can be administered without causing adverse effects [5]. *B. bifidum* has been shown to have advantageous effects on human health and has been confirmed as a safe choice for cancer therapy [6]. A multitude of studies have reported the anticancer properties of CFS, thereby promoting homeostasis and mitigating intestinal inflammation [7–10].

CFS derived from *B. bifidum* demonstrates sufficient potential for the treatment and prevention of gastrointestinal disorders. However, its effectiveness in reaching the target site is limited by adverse conditions in the gastrointestinal tract, including low pH and specific enzymes [11]. Therefore, the selection of an appropriate carrier is imperative for achieving optimal antitumor treatment outcomes. Recent research has focused on the utilization of nanoparticles, including those composed of chitosan, in the treatment of cancer [12]. Chitosan is a biocompatible and biodegradable polysaccharide derived from chitin. Chitosan has demonstrated antineoplastic properties, including the inhibition of tumor growth by inducing apoptosis, preventing angiogenesis, and inhibiting metastasis in cancer cells [13].

In a research study conducted by Wu et al. in 2024, it was demonstrated that chitosan nanoparticle (CTNP) enclosed with poly-L-glutamic acid can enhance targeted drug delivery and promote increased drug absorption by tumor cells [14]. Consequently, it can be regarded as a promising candidate for the treatment of colorectal cancer, exhibiting a notable anti-tumor effect. As indicated by several studies, the use of chitosan alone [15], chitosan loaded with protamine [16], and N-succinyl [17] has the potential to modulate the expression of genes involved in signaling pathways in various cancers, thereby impeding cancer progression.

It is important to note that several pathways are involved in the development of CRC, including Wnt/β-Catenin (*β-Catenin*), Phosphatidylinositol 3-kinase (*PI3K*) (*PTEN*), transforming growth factor (*TGF-β*), and Notch (*HES1*), Hedgehog (SHH) (*Bcl2*, *Gli2*), and Epidermal growth factor receptor (EGFR) (*TGF-α*), and genes such as interleukin 6 (*IL-6*), Toll-like receptor 4 (*TLR4*), and carcinoembryonic antigen (*CEA*) [18]. These pathways play an important role in the pathogenesis and metastasis of cancers. To date, however, no study has reported the effect of CTNP conjugated with cell-free culture medium of *B. bifidum* (B.b-sup) on CRC signaling pathways. The objective of this research was to investigate the effect of chitosan nanoparticles conjugated with cell-free culture medium of *B. bifidum* (CTNP/B.b-sup) on the expression of certain genes involved in CRC progression using Real-time PCR.

Materials and Methods

Preparation of the cell free supernatant of *B. bifidum*

The *B. bifidum* strain was obtained from the collection of the bacteriology department at Tarbiat Modares University of Medical Sciences (Tehran, Iran). The bacterial culture was carried out on de Man, Rogosa, and Sharpe agar medium (Merck, Germany) under anaerobic conditions using a gas pack (Merck, Germany) and jar at 37 °C for 72 h. Subsequently, the B. bifidum strain was sub-cultured in Brain Heart Infusion (BHI) broth (Merck, Germany) under anaerobic conditions for 24 h. The optical density (OD) of the bacterial suspension was then equated with the standard 0.5 McFarland, which contains 10^8 colony-forming units (CFU) per milliliter (mL). Subsequently, the bacterial suspension was subjected to a centrifugal process at 10,000 rpm for a duration of 10 min. CFS was prepared by using a 0.22-µm filter. The final step in the procedure entailed culturing the CFS on MRS agar medium under both aerobic and anaerobic conditions [19]. This was done to ensure the absence of any additional bacterial species and to confirm the CFS.

Preparation and characterization of CTNP/B.b-sup and CTNP

The ionic gelation method was employed to produce CTNP/B.b-sup and CTNP. The fabrication of CTNP involved the combination of 500 µL of tripolyphosphate (TPP) (Sigma, Germany) at a ratio of 1:4 with 1500 µL of phosphate-buffered saline (PBS) 1X solution (Gibco,

USA). The mixture was then added to 5 mL of a solution of chitosan at a ratio of 2:5, employing a gentle magnetic stirrer to ensure thorough integration. The mixture was subjected to complete mixing under sterile conditions for 1 h at room temperature. In order to produce CTNP/B.b-sup, a mixture containing 500 µL of TPP and 1,500 µL of B.b-sup at a ratio of 1:4 was prepared. Subsequently, the prepared mixture was introduced into the chitosan solution in a proportion of 2:5, gently and carefully, over a volume of 5 mL. The mixture was stirred under gentle conditions for 1 h at 25 °C. Subsequently, the pH was adjusted within the range of 8–9, and the mixture was subjected to centrifugation at 16,000 rpm at 4 °C for a duration of 30 min. Finally, the upper layer (supernatant) was removed, and the pellet containing the synthesized nanoparticles was collected.

The prepared nanoparticles were then lyophilized and stored at −20 °C for subsequent use. To investigate the morphological properties, zeta potential (ZP), and size distribution of CTNP/B.b-sup and CTNP, DLS (Dynamic Light Scattering) and TEM (Transmission Electron Microscopy) techniques were used [20].

Measurement of loaded protein on CTNP/B.b-sup and encapsulation efficiency (EE %)

The total protein within B.b-sup and the unloaded protein after CTNP/B.b-sup formation was assayed using the BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) at 572 nm. The protein loaded on CTNP/B.b-sup was determined indirectly by assessing the difference between the total of initial protein in B.b-sup and the amount of unloaded protein after the formation of CTNP/B.b-sup. Furthermore, the EE% of CTNP/B.b-sup was determined by employing the following formula [21].

$$Encapsulation\ efficiency\ (EE\ \%) = a - b/a \times 100$$

a: total protein in initial B.b-sup, b: free protein in B.b-sup after CTNP/B.b-sup formation

Release study

The protein released from CTNP/B.b-sup was assessed for 48 h under various pH conditions, including 6.8, 7.4, and 1.8, utilizing a BCA assay kit. The protocol involved the dissolution of CTNP/B.b-sup in 5 mL of PBS 1X solution and subsequent incubation at 37 °C. At predetermined time intervals of 2, 6, 12, 24, and 48 h, 25 µl of the CTNP/B.b-sup-containing PBS 1X solution were withdrawn and substituted with an equal volume of fresh PBS 1X. These samples were then subjected to protein concentration evaluation. Finally, the release of protein was calculated by following equation.

$$Release\ (\%) = a/b \times 100$$

a: released protein at a time point, b: loaded protein on CTNP/B.b-sup.

Caco-2 cells preparation

The human colorectal adenocarcinoma (Caco-2) cells were procured from the Pasteur Institute of Iran (Tehran, Iran). The cells were then cultivated in DMEM (high-glucose Dulbecco's Modified Eagle's Medium) (Sigma, Germany) containing 10% FBS (fetal bovine serum) (Gibco, USA), 1% penicillin–streptomycin (Gibco, USA), and 2% L-glutamine (DNA Biotech-Iran). The cells were then placed in a humidified incubator containing 5% CO2 at 37 °C for 48 h. Sub-culturing of the cells was initiated upon reaching 80–90% confluency. The cells were detached from a T75 culture flask (SPL, Korea), and 1 mL of 0.25% Trypsin–EDTA (Gibco, USA) enzyme was added to it. The cells were then maintained in an incubator containing 5% CO2 at 37 °C for 15 min. Subsequently, the contents of the flask were transferred into a falcon tube and subjected to a centrifugal process at 3,000 rpm for a duration of 5 min at a temperature of 4 °C. Following this, the upper layer of the sample was removed, and 1 mL of DMEM was added to the cell pellet. The cell pellet was then resuspended in a pipette, yielding a cell suspension suitable for sub-culturing in a new flask [22].

Evaluation of viability of Caco-2 cells after interaction with CTNP/B.b-sup

The cytotoxic effects of CTNP, B.b-sup, and CTNP/B.b-sup on Caco-2 cells were assayed using MTT solution (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide) (DNA Biotech, Iran). In summary, 1×10^3 cells were seeded in each well of a 96-well culture plate (SPL, Korea) and maintained for 48 h at 37 °C until reaching approximately 80% confluency. Subsequently, the cells were subjected to treatment with 100 µL of specific concentrations, including CTNP (0.05%), B.b-sup (1200 µg), and CTNP/B.b-sup (1200 µg ± 0.05%). Untreated Caco-2 cells and BHI broth medium were used as controls in all Caco-2 cell experiments including Viability assays. The BHI broth as control group was selected to evaluate the probable toxicity of bacterial culture medium on Caco-2 cells.

The microplates were then subjected to an incubation period of 24 h at a temperature of 37 °C. Following this, the culture medium was removed and 100 µL of MTT reagent was added to each well. The plates were then incubated for 4 h at 37 °C in the dark. Thereafter, the MTT solution was aspirated from each well and 100 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) was added to dissolve the formazan product. Subsequently,

the optical absorption of the resulting purple solution was measured at 570 nm using an microplate reader (BioTek, USA) [23].

RNA extraction and complementary DNA (cDNA) synthesis

The total RNA extraction from Caco-2 cells was conducted in accordance with the protocol provided by the manufacturer of the RNA Miniprep Super Kit (Bio Basic, Canada). In summary, 2×10^4 Caco-2 cells were seeded in a 6-well culture microplate (SPL, Korea) and maintained at 37 °C with 5% CO2 for 48 h. Following the attainment of 80% confluency, the culture medium was removed, and the cells were treated with 1 mL of serum-free DMEM lacking antibiotics, containing CTNP at 0.05%, B.b-sup at 1200 µg, and CTNP/B.b-sup at 0.05% + 1200 µg for a 24 h incubation at 37 °C with 5% CO2. In this study, RNA was extracted from both treated and untreated (as control) Caco-2 cells. The optical density of the extracted RNA was measured at 260/280 nm to assess the purity and concentration of the extracted RNA. Subsequently, the process of synthesizing cDNA was initiated using RNA samples exhibiting an optical density (OD) of approximately 1.8–2, in accordance with the protocol provided by the kit manufacturer (Yekta Tajhiz Azma, Iran). The synthesis of cDNA was further facilitated by a mixture containing 1 µL of random primer, 6 µL of template RNA, and 6.5 µL of DEPC-treated water. The mixture was subsequently subjected to a centrifugal process at a speed of 3,000 rpm for a duration of five minutes. Thereafter, the mixture was subjected to an incubation at 70 °C for a duration of 5 min. Thereafter, 5× first strand buffer (4 µL), dNTPs (1 µL), RNase (0.5 µL; 40 U/µL), and M-MLV (1 µL) were added to the mixture. The mixture was then subjected to incubation at 42 °C for a duration of 60 min, followed by heating at 70 °C for an additional 5 min.

Evaluation of expression of oncogenes and suppressors involved in CRC progression by Real-time PCR assay

In the present study, real-time PCR was conducted using an ABI real-time PCR System (Applied Biosystems, USA) to evaluate the effects of CTNP, B.b-Sup, and CTNP/B.b-Sup on the expression of genes associated with CRC signaling pathways in Caco-2 cells. The total reaction volume was 20 µL, containing 0.1 µM reverse primer, 0.1 µM forward primer, 6 µL RNase-free water, 2 µL cDNA, and 10 µL SYBR Green master mix (Amplicon, Denmark). The primer sequences employed to amplify the cDNA of these genes are provided in Table 1. The Glutaldehyde3-Phosphate Dehydrogenase (GAPDH) gene was utilized as an internal control. The relative quantification was performed using standard $2^{-\Delta\Delta CT}$ calculations, in which ΔCT represents the difference between the CT values of the target gene and the calibrator [24].

Untreated Caco-2 cells and BHI broth medium were used as controls in all Caco-2 cell experiments including gene expression assays. The BHI broth as control group was selected to evaluate the probable effect of bacterial culture medium on genes expression.

Statistical analysis

All experiments were done in triplicate and values are presented as mean ± standard deviation (SD). Data were analyzed using GraphPad Prism version 8 with one-way ANOVA and Tukey comparison test. p-value < 0.05 was considered significant.

Table 1 The sequence of primers used in real-time PCR

Oncogenes/Suppressors	Primers		
	Primer-F	Primer-R	reference
Bcl2	TGGAGAGTGCTGAAGATTGA	GTCTACTTCCTCTGTGATGTTGTAT	[25]
β-Catenin	CTGCTGTTTTGTTCCGAATGTC	CCATTGGCTCTGTTCTGAAGAGA	[26]
IL-6	GTCAACTCCATCTGCCCTTCAG	GGTCTGTTGTGGGTGGTATCCT	[27]
PI3K	TTGTCTGTCACACTTCTGTAGTT	AACAGTTCCCATTGGATTCAACA	[28]
TGF-α	ATGGTCCCCTCGGCTGGACAG	GACCACTGTTTCTGAGTGGCA	[29]
TGF-β	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA	[30]
CEA	TTACCTTTCGGGAGCGAACC	TGTTGCTGCGGTATCCCATT	[31]
TLR4	TTTCCTGCAATGGATCAAGGA	TTATCTGAAGGTGTTGCACATTCC	[32]
GLi2	AAGTCACTCAAGATTCCTGCTCA	GTTTTCCAGGATGGAGCCACTT	[33]
HES1	TCAACACGACACCGGATAAAC	GCCGCGAGCTATCTTTCTTCA	[34]
PTEN	CAAGATGATGTTTGAAACTATTCCAATG	CCTTTAGCTGGCAGACCACAA	[35]
Caspase9	GCAGGCTCTGGATCTCGGC	GCTGCTTGCCTGTTAGTTCGC	[36]

Results

Characterization of CTNP and CTNP/B.b-sup

As demonstrated in Fig. 1A and B, the size dispersion of CTNP and CTNP/B.b-sup was determined to be 421.7 ± 223.4 and 453.1 ± 230.8 nm, respectively. The zeta potential of CTNP and CTNP/B.b-sup were recorded at 11 ± 6.4 and 9.11 ± 3.6 mV, respectively (see Fig. 1C and D). These findings are further detailed in Table 2. As demonstrated in Fig. 2, TEM technique was utilized to examine the surface morphology of CTNP and CTNP/B.b-sup. The TEM images revealed that the morphology of CTNP is round and that its size is smaller than that of CTNP/B.b-sup (Fig. 2A). Following the loading of B.b-sup onto CTNP/B.b-sup, the nano-drug exhibited an augmented size (Fig. 2B).

Determination of loaded protein and EE % of CTNP/B.b-sup

As demonstrated in Fig. 3A, the total protein content within B.b-sup and the unloaded protein following CTNP/B.b-sup formation was determined to be 1500 and 300 µg/mL, respectively. Subtracting the unloaded protein from the total protein yielded a loaded protein content of 1200 µg/mL on CTNP/B.b-sup.

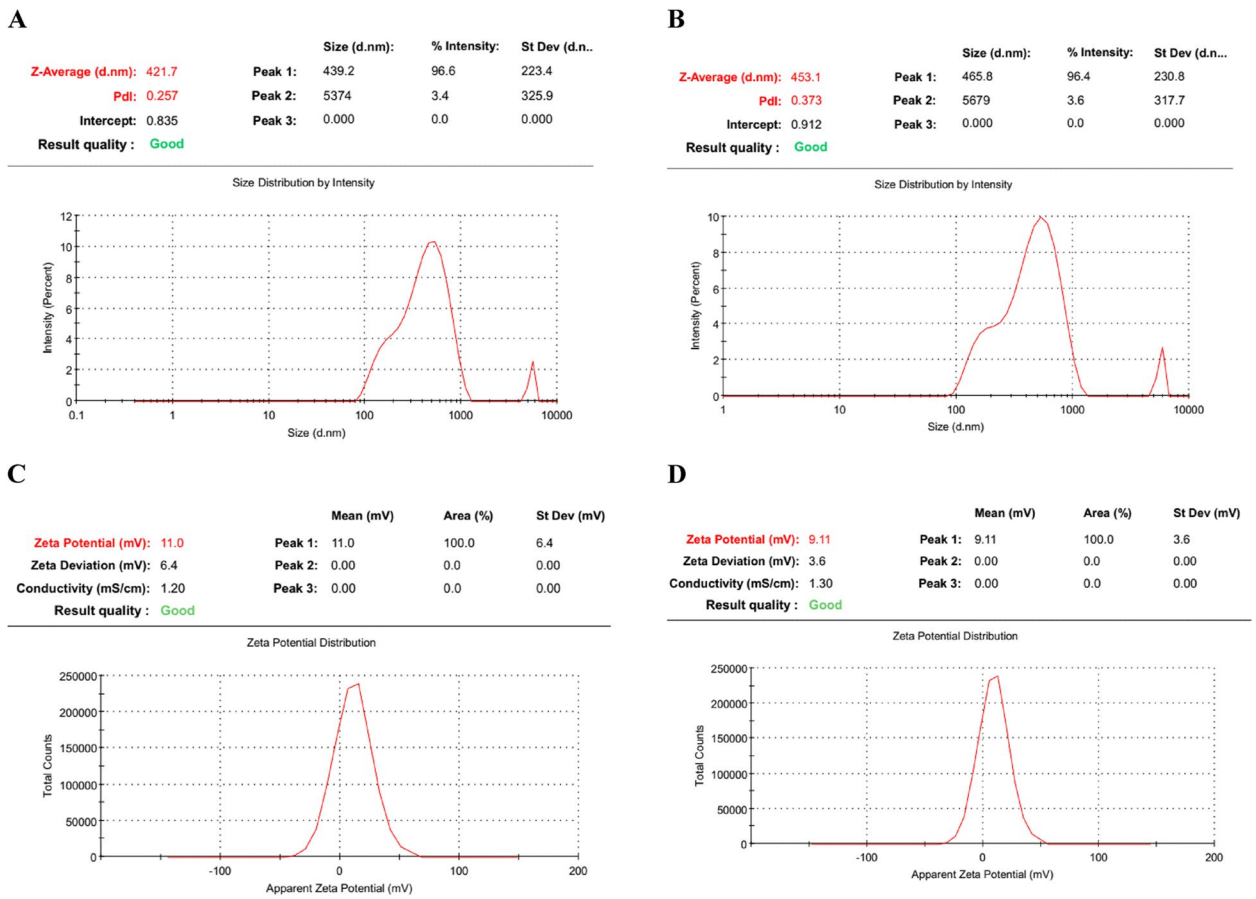

Fig. 1 Characterization of nanoparticles using DLS method. Average size was shown for CTNP (**A**) and CTNP/B.b-sup (**B**), and Zeta potential of CTNP (**C**) and CTNP/B.b-sup (**D**)

Table 2 The average size and zeta potential of CTNP and CTNP/B.b-sup

Formula	Zeta potential (mV)	Particle size (nm, mean ± SD)	Chitosan concentration %	EE% (Mean ± SD)	PDI
CTNP	11 ± 6.4	421.7 ± 223.4	0.05	-	0.257
CTNP/B.b-sup	9.11 ± 3.6	453.1 ± 230.8	0.05	83.3%	0.373

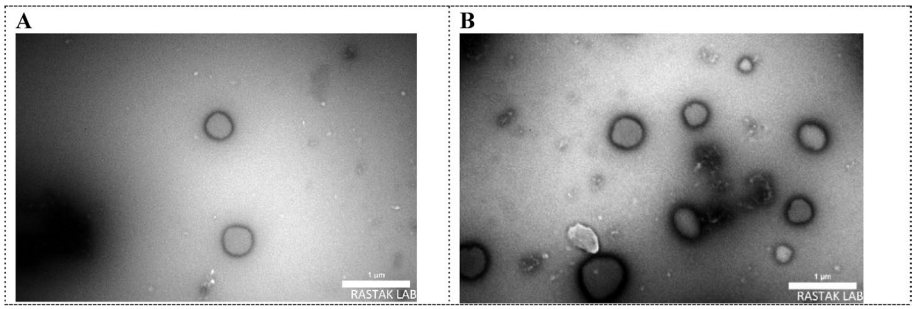

Fig. 2 Transmission electron microscopy (TEM) images were shown. TEM image of CTNP (**A**), CTNP/B.b-sup (**B**)

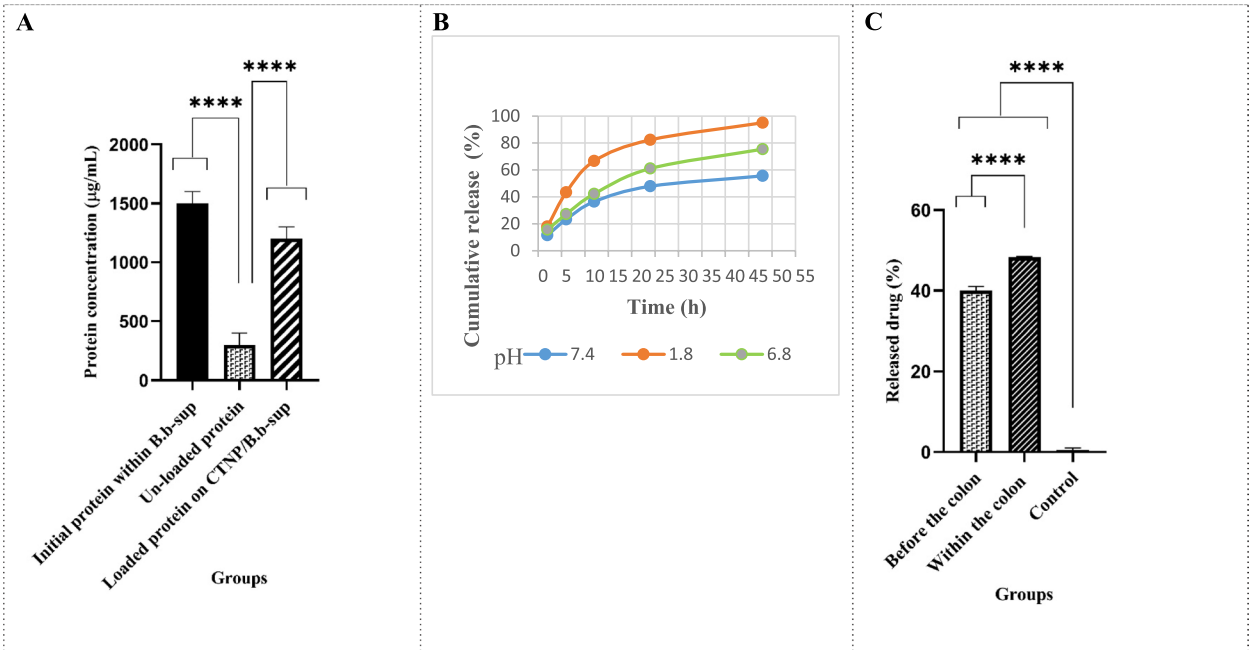

Fig. 3 Protein loading and release assay. Determination of the amount of loaded protein on CTNP/B.b-sup (**A**), the results indicated a significant difference with ****$p < .0001$. Release of protein from CTNP/B.b-sup determined at three pH (1.8;red color) (6.8; green color), and. (7.4; blue color) in time interval 2, 6, 12, 24, and 48 h (**B**). A one-way ANOVA analysis and Tukey's multiple comparisons test was conducted to examine the release of the drug in time intervals that correspond to the time preceding its arrival in the colon and the time during its presence within the colon (**C**). The results indicated a significant difference with ****$p < .0001$

In order to perform a valid comparison between the amount of protein within B.b-sup and CTNP/B.b-sup, B.b-sup was diluted with sterile PBS 1X. In this study, B.b-sup was utilized at a final concentration of 1200 µg/mL. The encapsulation efficiency of B.b-sup within CTNP/B.b-sup was found to be 83.3%, as presented in Table 2. This finding indicates that a significant quantity of B.b-sup was loaded onto CTNP/B.b-sup.

The release of protein from CTNP/B.b-sup

As demonstrated in Fig. 3B, the protein released from CTNP/B.b-sup was measured at three pH values (6.8, 7.4, and 1.8) at time intervals of 2, 6, 12, 24, and 48 h. At these time intervals, the protein released from CTNP/B.b-sup at a pH of approximately 6.8 (a value comparable to the colon pH) was found to be 15.7, 27.3, 42.3, 61.1, and 75.5%, respectively. Specifically, the percentage of protein released from CTNP/B.b-sup at pH ~ 6.8 (similar to the colon pH) was 75.5%, after 48 h. As shown in Fig. 3C, the protein released from CTNP/B.b-sup under conditions analogous to the stomach (pH ~ 1.8) was found to be 18% and 43.4%, at 2 and 6 h intervals, respectively. The released content at pH ~ 1.8 (similar to stomach conditions) is less than 40% after 5 h. A statistical analysis was conducted to examine the release of the drug in time intervals that correspond to the time preceding its arrival

in the colon and the time during its presence within the colon. It can be deduced from the available data from a statistical perspective, CTNP/B.b-sup will retain a significant proportion of its cargo until it reaches the colon, at which point it should reach its destination. The data demonstrate that pH 6.8 (colon-like conditions) is a suitable pH for protein release, and the highest rate of protein release from CSNP/L.a-sup was detected after 48 h.

Viability of Caco-2 cells after exposure with CTNP/B.b-sup

As shown in Fig. 4, following treatment of Caco-2 cells with CTNP (0.05%), B.b-sup (1200 µg), CTNP/B.b-sup (1200 µg +0.05%), and BHI broth, the viability of cells was 86.3, 86.2, 90.3, and 92%, respectively. The cell survival rates against CTNP, B.b-sup, and CTNP/B.b-sup were detected to be above 80%.

Effect of CTNP/B.b-sup on the expression of genes associated with CRC signaling pathways

The expression levels of *β-Catenin*, *PI3K*, *TGF-α*, *Bcl2* (see Fig. 5A-D), *TLR4*, *CEA*, and *TGF-β* oncogenes were found to be significantly reduced by CTNP/B.b-sup in comparison to untreated Caco-2 cells (utilized as a negative control).

Conversely, a significant increase was observed in the expression of *Gli2* and *HES1* oncogenes (see Fig. 6A and B). Following the treatment of Caco-2 cells with CTNP/B.b-sup, a significant decrease in the expression of *TLR4* and *CEA* genes was detected by 0.88 and 0.69-fold, respectively (Fig. 6C and D) ($p < 0.0145$ and $p < 0.0004$), while both CTNP and B.b-sup had no significant effect on the expression of these two genes individually. As demonstrated in Fig. 7A, the interaction of Caco-2 cells with CTNP led to a substantial decrease in *IL-6* oncogene expression. Conversely, B.b-sup was observed to induce a significant increase in this gene's expression ($p < 0.0001$). The findings suggest that the combination of CTNP and B.b-sup exerts no substantial influence on *IL-6* gene expression. This outcome is attributed to the neutral effect resulting from the conjugation of CTNP with B.b-sup.

Furthermore, CTNP and CTNP/B.b-sup have been demonstrated to elicit a substantial decline in the expression of the *TGF-β* oncogene ($p < 0.0001$), while B.b-sup has been shown to exert no significant effect on the expression of this gene (Fig. 7B). The findings indicate that the suppressive effect of CTNP/B.b-sup on the expression of the *TGF-β* gene is associated with CTNP. Furthermore, none of CTNP, B.b-sup, and CTNP/B.b-sup exhibited a significant effect on the expression of the oncogene *PTEN* (Fig. 7C), while they significantly increased the expression of the suppressor gene *caspase9* (Fig. 7D) ($p < 0.0004$ and $p < 0.0001$). Moreover, CTNP/B.b-sup exhibited the greatest reduction in the expression of the oncogene *TGF-α,* with a fold change of 0.03, and the highest increase in the expression of the suppressor gene *caspase9*, with a fold change of 73.4. Collectively, CTNP/B.b-sup appeared to demonstrate superior efficacy in modulating the expression of genes related to CRC progression. Fold changes of mentioned genes manifested in Table 3.

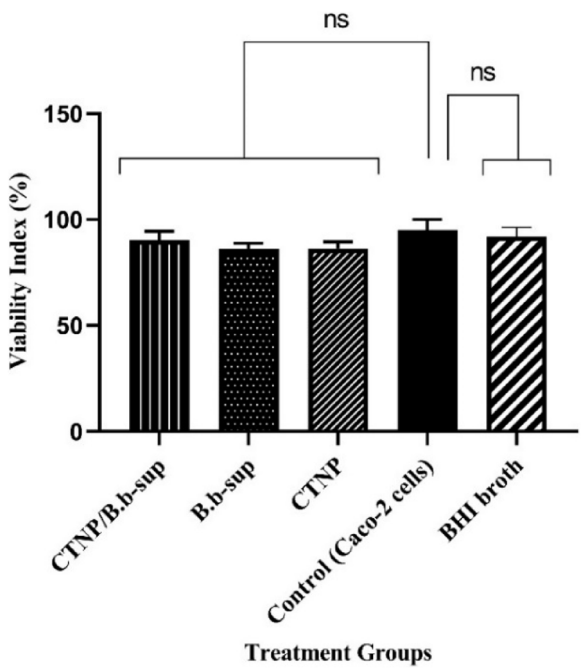

Fig. 4 Evaluation of viability of Caco-2 cells against CTNP, B.b-sup, and CTNP/B.b-sup using MTT assay (**B**). Untreated Caco-2 cells and BHI broth medium were used as controls in viability assay

Discussion

Colorectal cancer (CRC) is among the most prevalent malignancies worldwide, with a high mortality rate [37]. While there are advanced therapeutic options available for the treatment of CRC, these treatments often result in numerous adverse effects. The development of novel drugs has focused on the production of nanotherapeutics, given their ability to facilitate effective accumulation at the intended target site [38]. Consequently, biotherapy and nanotherapy are regarded as innovative therapeutic modalities for addressing CRC.

The specific mechanisms through which Bifidobacterium exerts its anticancer properties are not fully elucidated. However, it has been demonstrated that this bacterium is capable of producing short-chain fatty acids (SCFAs), which have been shown to impede the growth and maturation of cancer cells. Furthermore, the mass-produced SCFAs, namely butyrate, propionate, and

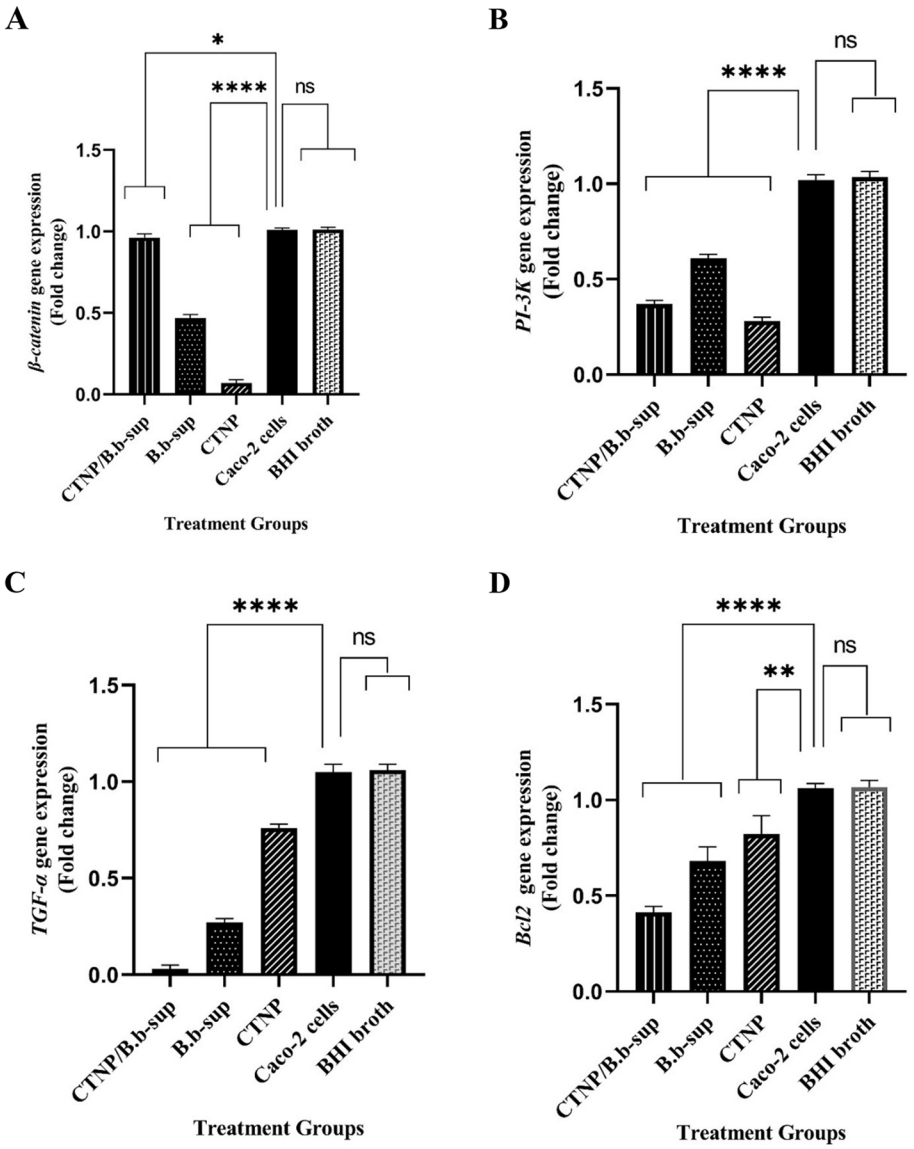

Fig. 5 Effects of CTNP, B.b-sup, and CTNP/B.b-sup on the expression of oncogenes related to CRC on Caco-2 cells using real-time PCR. Oncogenes including *β-Catenin* (**A**), *PI3K* (**B**), *TGF-α* (**C**), *Bcl2* (**D**). Values are presented as the mean ± SD of triplicate independent tests. * $p < .0291$, ** $p < .002$, and **** $p < .0001$ indicate statistically significant. Untreated Caco-2 cells and BHI broth medium were used as controls in all gene expression experiments

acetate, have been found to contribute to the alleviation of inflammation, a major risk factor in the development of cancer, thereby contributing to the regulation of gut homeostasis [8–10]. Furthermore, it has been demonstrated that bifidobacteria are capable of producing vitamin B12 and folates, which have been shown to have an anti-carcinogenic role [7].

The CTNP/B.b-sup demonstrated a substantial reduction in the expression of *β-Catenin*, *PI3K*, *TGF-α*, *Bcl2*, *TLR4*, and *CEA* genes, while concurrently exhibiting a significant increase in the expression of *caspase9* suppressor genes. It is hypothesized that the CTNP/B.b-sup exhibited the most pronounced inhibitory effect on the expression of the majority of the studied genes involved in CRC progression when compared to CTNP and B.b-sup individually.

This study showed that CTNP was more effective than B.b-sup and CTNP/B.b-sup in reducing *β-Catenin*, *PI3K* and *IL-6* gene expression. It is hypothesized that the improved efficacy of CTNP compared to CTNP/B.b-sup and B.b-sup is related to its enhanced cellular uptake due to its comparatively smaller size and greater positive

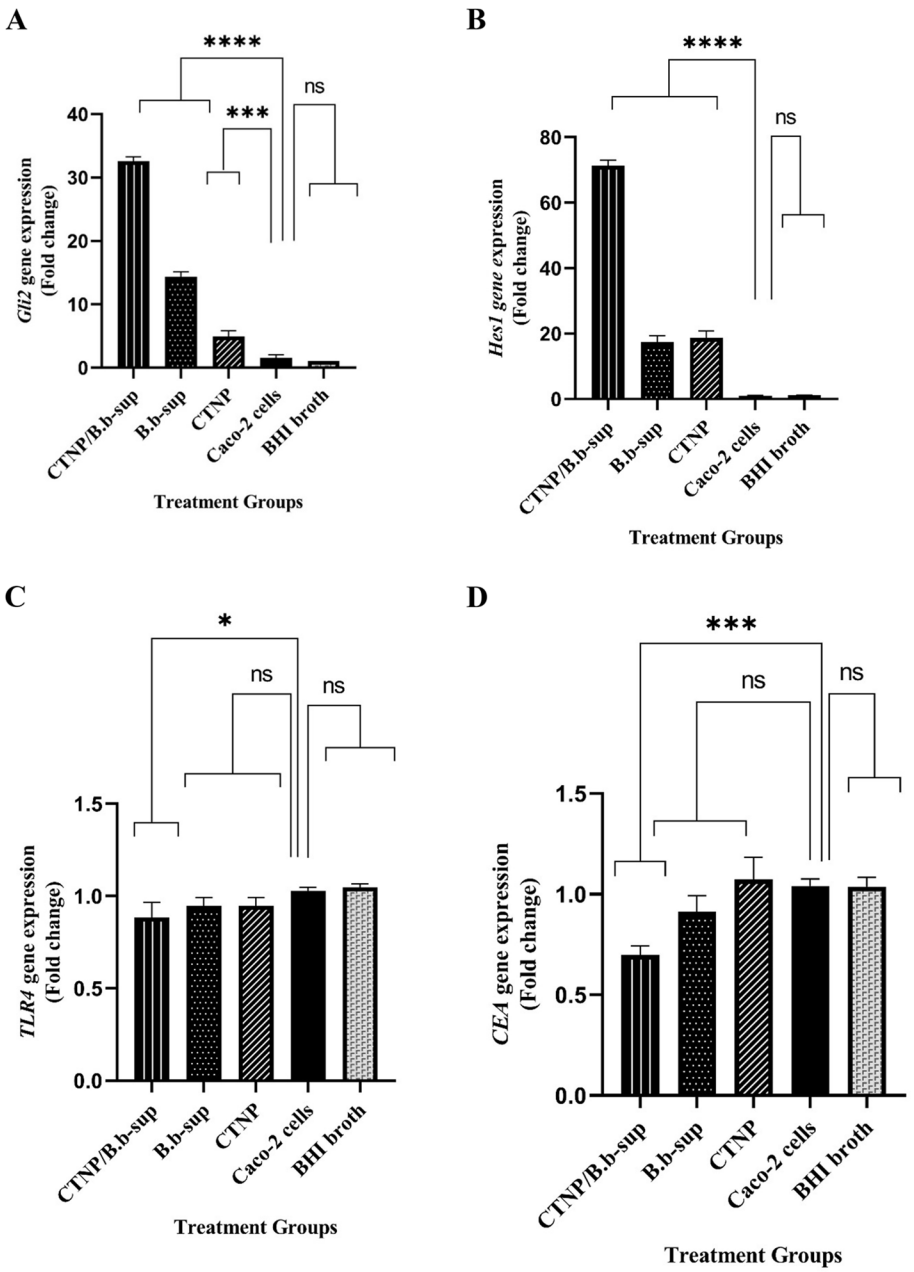

Fig. 6 Effects of CTNP, B.b-sup, and CTNP/B.b-sup on the expression of oncogenes related to CRC on Caco-2 cells using real-time PCR. Oncogenes including *Gli2* (**A**), *HES1* (**B**), *TLR4* (**C**), and *CEA* (**D**). Values are presented as the mean ± SD of triplicate independent tests. * $p < .0145$, *** $p < .0004$, and **** $p < .0001$ indicate statistically significant. Untreated Caco-2 cells and BHI broth medium were used as controls in all gene expression experiments.

charge [39]. Similarly, a study in Caco-2 cells showed that the uptake of CTNP in combination with other agents increased significantly for particle sizes between 200 and 600 nm. Conversely, absorption decreased at sizes above 600 nm [40].

Interleukin-6 (*IL-6*) is a pro-inflammatory cytokine that is regulated by transcription factors such as NF-κB and AP-1 (Activator Protein 1). Ultimately, *IL-6* activates Signal Transducer and Activator of Transcription 3 (STAT3). The activation of STAT3 plays a critical role in cancer development [27]. A recent study demonstrated that the administration of chitosan oligosaccharides (COS) led to a reduction in the levels of *IL-6* through two distinct signaling pathways. The first pathway was

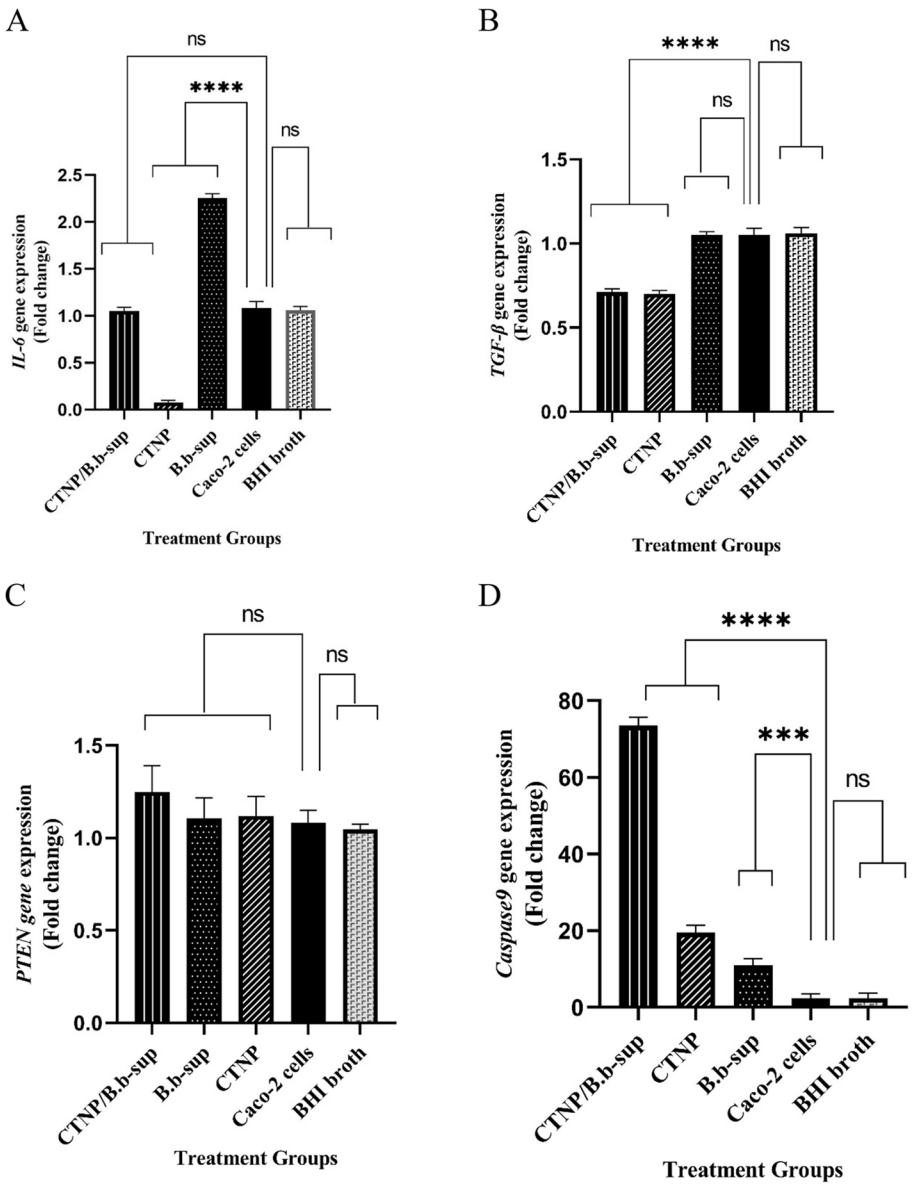

Fig. 7 Effects of CTNP, B.b-sup, and CTNP/B.b-sup on the expression of oncogenes and suppressors related to CRC. The genes including *IL-6* (**A**), *TGF-β* (**B**), *PTEN* (**C**), and *caspase9* **(D)**. Values are presented as the mean ± SD of triplicate independent tests. ***$p < .0004$ and ****$p < .0001$ indicate statistically significant. Untreated Caco-2 cells and BHI broth medium were used as controls in all gene expression experiments

dependent on the NF-κB-mediated ERK1/2 pathway, while the second pathway was independent of NF-κB and involved the p38 MAPK pathway [41]. Our findings corroborate the conclusions of previous studies that CTNP can inhibit *IL-6* gene expression. A notable finding of the present study was that CTNP, when administered individually, resulted in a significant decline in *IL-6* gene expression ($p < 0.0001$). However, the combination of CTNP and B.b-sup exhibited no significant impact on *IL-6* gene expression. The results of the study showed that the suppressive effects of CTNP were counteracted by B.b-sup, resulting in a null effect on *IL-6* gene expression by the innovative CTNP/B.b-sup nanodrug.

A study by Fahmy et al. (2019) demonstrated that *Bifidobacterium longum* effectively reduced *IL-6* levels in a mouse model of CRC, leading to an amelioration of carcinogenic lesions in colon cells [42]. The results of previous and current studies suggest that different Bifidobacterium species exert different effects on gene expression associated with CRC signaling pathways.

The release of transforming growth factor-β (TGF-β) by cancer cells, as well as by stromal fibroblasts and other

Table 3 Fold change of oncogenes and suppressors associated with CRC signaling pathways

Oncogenes	Fold change		
	CTNP	B.b-sup	CTNP/B.b-sup
B-Catenin	0.07	0.47	0.96
PI3 K	0.28	0.61	0.37
IL-6	0.08	2.25	1.05
TGF-a	0.76	0.27	0.03
Bcl2	0.82	0.68	0.41
TLR4	0.94	0.94	0.88
CEA	1.07	0.91	0.69
TGF-β	0.70	1.05	0.71
Gli2	4.91	14.32	32.5
HES1	18.61	17.38	71.32
Suppressors	Fold change		
	CTNP	B.b-sup	CTNP/B.b-sup
PTEN	1.11	1.10	1.24
Caspase9	19.49	11.01	73.4

cells present in the tumor microenvironment, has been demonstrated to promote the progression of cancer. This progression is achieved by influencing the structure of the tumor and by inhibiting the anti-tumor function of immune cells. Consequently, the suppression of TGF-β signaling has emerged as a critical requirement and a major focus for improving the efficacy of immuno-oncology therapies [43].

The process of binding of *TGF-β* to its receptors initiates a series of signals, involving the phosphorylation of SMADs (maternal suppressors against decapentaplegic). This process leads to the formation of SMAD complexes, which are subsequently translocated to the nucleus. In the nucleus, these complexes interact with specific DNA sequences, thereby regulating the transcription of diverse target genes. In addition, TGF-β receptors have been shown to regulate Smad-independent pathways, which have been found to be critical for the modulation of both Smad signaling and Smad-independent TGF-β responses [44]. It has been posited that CTNP/B.b-sup exerts its antineoplastic effects by modulating *TGF-β* gene expression, thereby probably influencing the transcription of genes and resulting in suppression of colon cancer cells.

The *CEA* (carcinoembryonic antigen) has been shown to possess both the capacity to inhibit cell death and the ability to upregulate cell adhesion molecules associated with metastasis. In addition, it has been observed to interact with the type I receptor of *TGF-β* (TBRI), thereby altering the *TGF-β* signal pathway and enhancing tumor cell proliferation [45]. This research also confirmed that CTNP/B.b-sup can inhibit *CEA* gene expression and suppress colon cancer cell progression, probably by interfering with the *TGF-β* signaling pathway.

Apoptosis, a process characterized by cell death, occurs through two main pathways: the mitochondria-dependent (intrinsic pathway) and the death receptor-dependent (extrinsic pathway) [46]. In a recent study, Faghfoori et al. (2021) demonstrated that *B. bifidum* secretome, a complex mixture of bioactive substances released by the bacterium, can significantly inhibit colon cancer cells by downregulating anti-apoptotic proteins, such as *Bcl2*, in Caco-2 and HT-29 cells compared to normal epithelial cells (KDR/293) [4].

In a separate study, the suppression of proteins involved in apoptosis, including Bcl-2, was observed in human umbilical vein endothelial cells (HUVECs) by the action of a chitosan oligosaccharide [47]. The findings of this study demonstrated that CTNP/B.b-sup exerted a substantial inhibitory effect on Bcl-2 gene expression in Caco-2 cell lines. This observation indicates that CTNP/B.b-sup may potentially trigger the intrinsic apoptotic pathway through the reduction of *Bcl2* expression.

It is noteworthy that CTNP/B.b-sup demonstrated a more pronounced efficacy in enhancing *caspase9* gene expression in comparison to CTNP and B.b-sup, underscoring a synergistic effect. *Caspase9* plays a pivotal role in the activation of intrinsic apoptosis, which is a mitochondrial-dependent form of programmed cell death [48]. Consequently, the CTNP/B.b-sup formulation, utilized in the present study, which overexpressed the pro-apoptotic proteins such as *PTEN*, is probably capable of inducing apoptosis in colon cancer cells likely through the intrinsic apoptosis pathway.

The findings of this study indicate that CTNP/B.b-sup does not induce toxic effects in Caco-2 cells. Furthermore, the results demonstrate a substantial release of B.b-sup from nanoparticles at a pH of approximately 6.8, which is analogous to the pH of the colon, within a 48 h period. In light of these observations, the CFS utilized in this investigation emerges as a safe alternative for the treatment of colorectal cancer (CRC). When used in conjunction with CTNP as a suitable drug delivery system, the CFS can serve as a promising strategy in the context of CRC management.

A multitude of studies have substantiated that CTNP functions as a delivery system, exerting a plethora of influences on signaling pathways associated with cancers when administered in conjunction with various agents, including insulin-like growth factor 1 receptor, folic acid, and Herceptin [13]. This study underscores the synergistic impact of CTNP and B.b-sup on multiple genes implicated in CRC signaling pathways, thereby substantiating the notion that CTNP/B.b-sup

holds considerable promise as a therapeutic modality for the management of CRC.

Conclusion

In conclusion, the primary findings of this study are as follows: i) CTNP/B.b-sup significantly reduced the expression of several tumor oncogenes, including *β-Catenin*, *PI3K*, *TGF-α*, *TLR4*, *CEA*, *TGF-β*, and *Bcl2*. ii) CTNP/B.b-sup could cause a significant decrease in the expression of the *TGF-β* oncogene, whitch its efficacy is supposed to be attributed to CTNP, iii) CTNP/B.b-sup exhibited superior efficiency in inducing *caspase9* gene expression, thereby highlighting its significant pro-apoptotic potential. It can be concluded that CTNP/B.b-sup is a suitable drug delivery system with anticancer properties. This can be regarded as a complementary therapeutic in clinical settings.

Abbreviations

CRC	Colorectal cancer
B. bifidum	*Bifidobacterium bifidum*
USFDA	United States Food and Drug Administration
CFS	Cell free supernatant
CTNP	Chitosan Nanoparticles
PI3 K	Phosphatidylinositol 3-kinase
TGF-β	Transforming growth factor
SHH	Hedgehog pathway
EGFR	Epidermal growth factor receptor
IL6	Interleukin 6
TLR4	Toll-like receptor 4
CEA	Carcinoembryonic antigen
B.b-sup	Cell-free culture medium of *B. bifidum*
BHI	Brain heart infusion
OD	Optical density
TPP	Tripolyphosphate
PBS	Phosphate-buffered saline
CTNP/B.b-sup	Chitosan nanoparticles conjugated with cell free supernatant of Bifidobacterium bifidum
ZP	Zeta Potential
DLS	Dynamic Light Scattering
TEM	Transmission Electron Microscopy
EE	Encapsulation efficiency
Caco-2	Human colorectal adenocarcinoma
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovin serum
DMSO	Dimethyl sulfoxide
cDNA	Complementary DNA
GAPDH	Glutaldehyde3-Phosphate Dehydrogenase

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Authors' contributions
B.B. designed and supervised the work. R.M. performed the experiments. R.M, M.S, B.B, and FF contributed to data interpretation. R.M and M.S drafted the manuscript. All authors read and approved the final manuscript.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author with no restriction.

Declarations

Ethics approval and consent to participate
This project has been approved by the Ethic Committee of Tarbiat Modares University (code: IR.MODARES.REC.1401.253).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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