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The Combined Effect of D-peptide B and *Bifidobacterium bifidum* Lysate on the Expression of *MAP1LC3A* and *CASP3* Genes in Human Colorectal Adenocarcinoma Cells

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Abstract

In the realm of cancer research, novel approaches complementing conventional treatments are gaining prominence to improve patient outcomes. Antimicrobial peptides (AMPs), and bacteria, as emerging strategies, hold promise for cancer therapy. Recent studies suggested that the administration of peptides and bacteria may eliminate colorectal cancer (CRC) cells by triggering apoptosis, and autophagy processes, underscoring the potential of these compounds in CRC treatment. Using the human CRC cell line HT-29, we evaluated the combined effects of D-peptide B and *Bifidobacterium bifidum* lysate on the expression of the microtubule associated protein 1 light chain 3 alpha (*MAP1LC3A*), referred to as *LC3A* hereafter, and caspase 3 (*CASP3*) genes. D-peptide B and *B. bifidum* lysate were applied to control human embryonic kidney epithelial cell line HEK293 and HT-29 at their respective half-maximal inhibitory concentration (IC50) values. We performed total RNA extraction, complementary DNA (cDNA) synthesis, and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) with gel electrophoresis to examine changes in the gene expression. Tukey's test and GraphPad Prism One-Way ANOVA were used for the statistical study. The combined treatment of D-peptide B and *B. bifidum* lysate with HT-29 cells resulted in a considerable decrease in the vitality of cancer cells. Furthermore, when HT-29 cells were compared to control cells, the expression of *LC3A* and *CASP3* genes increased. The results point to the possibility of combining D-peptide B with *B. bifidum* lysate as a supplementary therapy for CRC. Furthermore, these findings may help improve our knowledge of the molecular processes driving apoptosis and autophagy, as well as their potential therapeutic implications in CRC.

Keywords: Colorectal cancer; Antimicrobial peptides; *Bifidobacterium bifidum*; *CASP3*; *LC3A*.

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Introduction

According to the World Cancer Research Fund, CRC was the second most prevalent cancer in women and the third most common cancer in men in 2020 (1). CRC was recognized as the second major cause of cancer-related deaths in 2022, and both worldwide incidence and mortality are anticipated to climb in the following years (2, 3). Over the last 25 years, CRC incidence and mortality have increased worldwide among individuals under 50 (2), but in developed countries, regular colon cancer screening has led to a decrease in CRC incidence and mortality. The routine treatment of CRC incorporates surgery, radiotherapy, and chemotherapy (4), which may have downsides, such as the lack of specific tumor selection, solubility, and drug resistance (5, 6). Chemotherapy drugs target cancer cells but may damage healthy cells nearby. Most current chemotherapies face resistance from CRC patients, diminishing the effectiveness of anticancer drugs, and eventually resulting in chemotherapy treatment failure (7). Despite the progress made in therapeutic procedures, the treatment of CRC remains challenging (7). The challenges are associated with its late detection at advanced stages, increasing the difficulty of successful treatment, potential metastasis to other organs, and the development of resistance to standard therapies like chemotherapy. Furthermore, genetic heterogeneity of CRC subtypes further complicates the customization of treatments to match the unique profile of each patient. Moreover, the substantial side effects associated with conventional treatments can affect a patient's quality of life.

Complementary and integrative treatments for CRC will not replace conventional medical treatments like surgery, chemotherapy, and radiation therapy. Instead, complementary and integrative therapies, along with standard medical care, help to manage symptoms, reduce side effects, and improve the overall well-being of patients. An essential complementary therapy includes implementing nutritional and dietary recommendations, which may include the use of vitamins and medications. Peptide therapy has great potential as a new approach for developing anticancer drugs due to its several advantages, including low toxicity, small molecular size, simple manufacturing process, and capacity to enter cell membranes (8). AMPs show a remarkable ability to selectively target and eliminate cancer cells. Certain AMPs have cytotoxicity against cancer cells in terms of their amino acid composition and cationic amphiphilic structure, which allows them to target cancer cell membranes while avoiding normal mammalian cells (8). Among AMPs, D-peptides, short synthetic enantiomeric peptides, are effective at causing cell death via the membrane depolarization, and apoptosis (9).

On the other hands, several bacteria including *B. bifidum* exhibit anticancer effects on CRC cells (10, 11). Such bacteria improve the environment within the tumor, stimulate various immune cells such as dendritic cells, helper T cells, natural killer cells, and cytotoxic T cells, and increase the production of immune cytokines that promote tumor growth, such as interleukin-2 and interferon (12). Bacterial lysates are often used in immunotherapy or as adjuvants to stimulate the immune system. Moreover, bacterial lysate can combine with the carcinogens in the gastrointestinal tract, reducing their quantity (13). Thus, using AMPs and bacterial lysate as complementary treatment at early-stage prognosis may significantly influence the development of CRC. Cancer treatment with natural compounds, such as peptides and bacterial lysate has various benefits, including potential induction of apoptosis and autophagy.

Autophagy and apoptosis are critical processes responsible for maintaining homeostasis. While apoptosis destroys damaged cells, autophagy recycles certain intracellular organelles and chemicals (14). Autophagy is facilitated by soluble protein *LC3A,* which is widely found in mammalian tissues and cell cultures. Caspases, including *CASP3*, a type of protease enzyme, play a critical role to regulate the apoptosis which is conserved across multicellular organisms. The products of *LC3A* and *CASP3* may induce cell death in cancer cells via the autophagic and apoptosis pathways, respectively. Conducting research is necessary to enhance and expand the existing techniques and investigate new and supplementary methods for treating CRC. D-peptide B has a low molecular weight, and peptides with lower molecular weight are preferred to larger molecules since high molecular weight peptides have a higher risk of anaphylactic shock *in vivo* (15). In addition, we opted to use bacterial lysate in this study rather than live bacteria since preliminary data suggests that bacterial lysates, which do not contain live microbes, may be a safer alternative to regulate the immune system without potential side effects (16, 17).

Studies suggest peptides and bacterial lysate as possible complementary therapies for CRC. To the best of our knowledge, however, no research was published looking at the anticancer effects of peptides, and bacterial lysate combined on HT-29 cells. Therefore, the aim of this work was to look into how D-peptide B and *B. bifidum* lysate in combination affected the expression of the *LC3A* and *CASP3* genes in HT-29 cells. The aim was pursued by specifying two objectives: (i) assessing the viability of HT-29 and control HEK293 cells after

exposure to D-peptide B, *B. bifidum* lysate, and their combination, and (ii) examining the expression of *LC3A* and *CASP3* genes in the treated cells. By conducting this study, we hope to further knowledge about the possible therapeutic uses of D-peptide B and *B. bifidum* in combination for the complementary treatment of CRC.

Materials and Methods

1. Bacterial culture and peptide stock preparation

The commercially synthesized peptide used, D-peptide B (RLRLRIGRR-NH_2) with 97.24% purity, was purchased (P200211-MJ495935, ProteoGenix, Schiltigheim, France). This peptide was delivered in powdered form which was subsequently reconstituted in sterile distilled water to create a solution with various concentrations for use in the experiment. The Iranian Research Organization for Science and Technology (Tehran, Iran) provided *B. bifidum* glycerol stock. The bacteria were grown in De Man, Rogosa and Sharpe (MRS) broth (i23117, Ibresco) in an anaerobic culture and incubated for 24 h at 37°C. After the incubation time, the culture was centrifuged for 2 min at 4°C at 9,000 g. The supernatant was discarded and the pellet (1 g) was soaked in 5 ml 1M phosphate buffered saline. A Hielscher, Germany, probe sonicator was used to lyse the cells. Then, a 22-micron syringe filter was utilized to filter the bacterial lysate and a spectrophotometer was then employed to measure the samples' optical density at a wavelength of 670 nm.

2. Cell lines and culture condition

The Pasteur Institute of Iran (Tehran, Iran) provided HT-29 and HEK-29 cell lines, which were supplied in a culture flask with active growth media. The first step was to culture cells in Dulbecco's Modified Eagle Medium High Glucose media (D6429, Sigma). The media contained 10% fetal bovine serum (FBS) (16000044, Gibco), and 100 units/ml penicillin, and 100 µg/ml streptomycin (10378016, Gibco). Once the main culture cell density reached 75%, passage was carried out. To neutralize the impact of trypsin, an aliquot (0.5 ml) of culture medium containing 10% FBS was introduced into each flask via pipette to generate a cell suspension. After transferring the suspension to 15-ml Falcon tubes, flasks were centrifuged at 25°C for 1.5 min at 120 rpm. Following this procedure, the supernatant was carefully removed and discarded. In order to get a uniform cell suspension, 1 ml of cell culture media containing 10% FBS was introduced to the cell granule using a pipette. Based on cell density, the resulting suspension was subsequently divided into two additional 25× 25 cm culture flasks. To each flask, 4 ml cell culture medium containing 10% FBS was added. Then, new containers were placed in the incubator. In order to ensure a consistent density of 70 - 80%, the culture medium was replaced every three days. The cells were gently aspirated and a small aliquot of the resulting cell suspension was dispensed onto a Neobar slide. The cell count was performed using a microscope. To attain an approximate confluence of 80%, cells were seeded into 24-well plates at a density of 1×10^4 cells per well. The plates were then incubated at 37°C for a duration of 24 h under a humidified atmosphere containing 5% CO_2.

3. Cell viability assay

A modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was employed to assess the effect of various concentrations of peptide, bacterial lysate, and their combination on the viability of HEK293 and HT-29 cells. MTT assay is a commonly used technique in cytotoxicity investigations because of its accuracy, speed, and relative simplicity (18). The experiment was conducted using an assay kit (BI-2004, Bio-idea) in accordance with the instructions provided with the kit. Absorbance measurements were used to determine the percentage of viable cancer cells remaining after the exposure to various concentrations of tested compounds. This measurement is then interpreted as the anticancer activity of the compound and its IC50 values. IC50, defined as the concentration of an inhibitor at which binding is reduced by half, signifies the dosage of a cytotoxic compound at which 50% viability is achieved via the MTT assay. The cell survival rate was determined by comparing IC50 values to the control cells, *i.e.*, untreated HT-29 and untreated HEK293 cells.

In brief, 1×10^4 cells were seeded in 96-well plates and treated with different doses of peptide, bacterial lysate, or their combination for 24 h. The concentration of peptide stocks, bacterial lysate, and their combination were as follows: peptide stock solution (µM): 75.00, 50.00, 30.00, 20.00, 5.00, and 1.00; bacterial lysate (mg/ml): 1.25, 1.00, 0.50, 0.25, 0.15, 0.10, and 0.05; peptide (µM) / bacterial lysate (mg/ml): 75.00 / 1.25, 50.00 / 1.00, 30.00 / 0.50, 20.00 / 0.25, 15.00 / 0.15, 5.00 / 0.10, and 1.00 / 0.05. After the incubation, each well received 10 µl MTT solution, and the plate was incubated for an additional 4 h. Following incubation time, 100 µl dimethyl sulfoxide (DMSO) was added to the wells to dissolve the pellet after the medium was carefully aspirated out. The plates were then shaken gently for 10 min. The optical density of every well was quantified at 570 nm using an ELISA reader with a 630

nm reference wavelength.

4. RNA extraction

Total RNA was extracted from HT-29 and HEK293 cells that had been treated with peptide, bacterial lysate, and their combination using a total RNA extraction kit (A10123150, Parstous, Tehran, Iran). An aliquot (1,000 µl) of each sample were transferred to RNase-free microtubes, and after two min centrifuging at 9,000 g, the supernatant was discarded. An aliquot (300 µl) of Lysis Buffer (RL Buffer) was added to each microtube mixed with pellet using a pipette. The mixture was kept at room temperature for 5 min. Each microtube was then filled with 150 µl chloroform, and slowly rotated 180 degree for 15 s. Microtubes were then left for 3 min at room temperature, followed by centrifugation at 13,000 g for 12 min at 4°C. After collecting and transferring RNA-containing supernatant into the fresh microtubes, 70% ethanol was added and mixed well. Following loading each prepared sample into a microtube, the liquid was removed from the columns and the sample was centrifuged for 1 min at 13,000 g. The liquid was then removed from the columns after 350 µl Wash Buffer (PW Buffer) was added and centrifuged at 13,000 g for 1 min. The columns were then inserted into fresh microtubes. After that, 50 µl RNase-free DEPC water was added, and the microtubes were left at room temperature for 3 min. A further centrifugation cycle lasting for 1 min at 13,000 g successfully extracted the RNA from the columns. Nanodrop (Thermo Fisher Scientific, U.S.) was used to confirm the amount and quality of total extracted RNA, and the microtubes containing the RNA were kept at -70°C for the next procedures.

5. Primer design and RT-qPCR

The reference gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and the sequences of *LC3A* and *CASP3* were acquired from National Center for Biotechnology Information in Tehran, Iran. Primer design was then completed with the use of Gene Runner software (version 6). The primers designed were as follows: *LC3A* Forward, TAAGGAGGTACAGCAGATC and Reverse, TCACCAGCAGGAAGAAGC (melting temperature, 54°C; product length, 200 bp); *CASP3* Forward, TGCTATTGTGAGGCGGTTGT and Reverse, ACACCCACCGAAAACCAGAG (melting temperature, 60°C; product length, 162 kb); *GAPDH* Forward, ACACCCACTCCTCCACCTTTG and Reverse, TCCACCACCCTGTTGCTGTAG (melting temperature, 62°C; product length, 112 bp). Easy cDNA synthesis kit (A101161, Parstous, Tehran, Iran) was used for cDNA synthesis. An aliquot (8 µl) of each pure RNA sample were transferred into microtubes, and then 2 µl enzyme mix (H-minus MMLV (thermostable), RNase inhibitor, stabilizer), and 10 µl buffer mix, 2× RT Buffer, dNTP Mix 1mM, MgCl2 8mM, Oligo d(t)16, random hexamer, stabilizer, (60.2020, Parstous, Tehran, Iran), were added. With the use of a vortex, the components were mixed and tubes were left at room temperature for five min. The microtubes were then placed into a PCR thermocycler, and the program was set up for cDNA synthesis. This included three steps: primer binding (10 min at 25°C), cDNA synthesis (60 min at 47°C), and enzyme inactivation (5 min at 85°C). Following process completion, the generated cDNA was stored at -20 °C.

At this stage, four cDNA samples were made, one for each of the following: cells treated with peptide, bacterial lysate, peptide and bacterial lysate combined, and the control cDNA sample. PCR procedure consisted of 40 consecutive cycles under the following conditions: 3 min at 95°C and 20 s at 95°C for DNA denaturation, 20 s at 53°C for primer annealing, and 10 s at 72°C for Taq DNA polymerase enzyme binding and extension. A melting curve analysis was carried out at the end of PCR, spanning a temperature range from 60°C to 95°C. Details regarding the precise quantity of compounds used in RT-qPCR are outlined in Appendix A. For visual presentation of data, two separate graphs were generated for each sample using Rotor-gene Q series by software QIAGEN. The main gene (*LC3A* or *CASP3*) was shown on one graph, while *GAPDH* was depicted on the other. For the PCR cycle diagrams, a threshold line was created in the program. The software then used the threshold line to identify the data and provide the Ct figures.

6. Amplified product analysis

The quantification of PCR product for *LC3A*, *CASP3* and *GAPDH* genes were defined using the threshold (Ct). Relative gene expression was assessed using the threshold cycle, which was then normalized with respect to *GAPDH* which is a housekeeping gene. ΔCt was used to determine the difference between the target and reference genes, and the 2-ΔΔct technique was used to compute Fold Changes. In order to confirm that certain gene segments were successfully amplified and to ensure that nonspecific products were not produced, gel electrophoresis was conducted. The amplified products from the target and reference genes, as obtained via RT-qPCR, were loaded onto a 2% agarose gel using a 10,000 – 250 bp ladder and subjected to electrophoresis.

7. Statistical analysis

Statistical analysis for MTT assay, and gene expression was conducted using One-Way ANOVA via GraphPad Prism 8. The results were presented as mean values, along with their corresponding standard deviations. Significance levels were indicated using different superscripts (*, **, ***, and ****) denoting statistically significant differences. When the p-value was less than 0.05, statistical significance was confirmed. In HEK293, and HT-29 cells, Tukey's test was performed to compare the expression of the *LC3A* and *CASP3* genes in each treated group to that of other treated groups. Various superscripts (**, ***, and ****) were used to signify various significance levels for statistically significant differences ($p < 0.05$).

Results

1. Cell viability and inhibitory effect analysis

MTT cell viability test was used to assess the effect of peptide, bacterial lysate, and their combination on HT-29 cell viability. HEK293 cells served as the control group in this comparison. Additionally, the inhibitory effects of peptide and bacterial lysate on normal cells (HEK293) and cancer cells (H-29) were investigated. At first, the survival rate for untreated HT-29 cells was 94.99% and for untreated HEK293 cells was 98.96%. Subsequently, the data for treated cells were normalized relative to their respective initial values. The significance of cell viability and inhibitory effect were assessed via statistical analysis using GraphPad Prism 8 software. The control data provides evidence of the inhibitory effects of D-peptide B on the normal cells, even at a lower concentration, *i.e.*, 1 µM, and this finding holds significance for future studies to take into account. Furthermore, when peptide and bacterial lysate are combined to treat cells, the inhibitory impact on normal cells has significantly increased. In general, the inhibitory impact on control cells is still less pronounced than that on cancer cells.

HT-29 cell viability significantly decreased when treated with peptide stocks at concentrations of 15, 20, 30, 50 and, 75 µM (Figure 1). While peptide showed cytotoxicity on HEK293 cells as well, its highest toxicity, observed at 75 µM, was statistically less significant than its effect on HT-29 cells. Furthermore, at a concentration of 50 µM peptide, HT-29 cells approached an approximate IC50 value. Consequently, 50 µM was selected as the concentration for viability assessment via the study. Similarly, when treated with bacterial lysate at dosages of 0.25, 0.5, 1, and 1.25 mg/ml, cell vitality in control cells (HEK293) remained greater, however cell viability in HT-29 cells declined significantly. At a bacterial lysate concentration of 0.5 mg/ml, the approximate IC50 value for HT-29 cells was obtained (Figure 2). Consequently, IC50 for HT-29 cells treated with bacterial lysate was set at 0.5 mg/ml. Cell viability in HT-29 cells was significantly reduced at all concentrations when treated with a combination of peptide and bacterial lysate (Figure 3). This combination showed a less significant cytotoxicity on HEK293 cells, notably up to 30 µM and 0.5 mg/ml. A

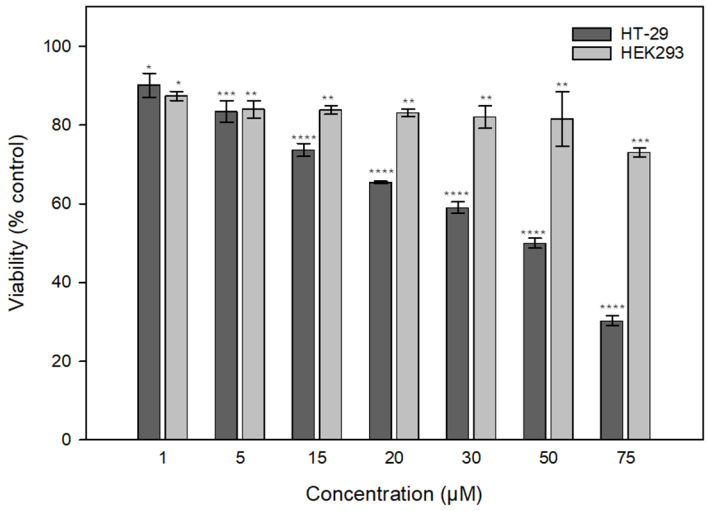

Figure 1. Cell viability of HT-29, and HEK293 cells treated with different concentrations of D-peptide B in 24 h. * shows the statistically significance of data achieved by One-Way ANOVA (*$p < 0.05$). All data shown represent the mean of at least two independent experiments ± SEM and significance was relative to the control. Bars represent fold differences of mean normalized % viability values ± SEM (n=4).

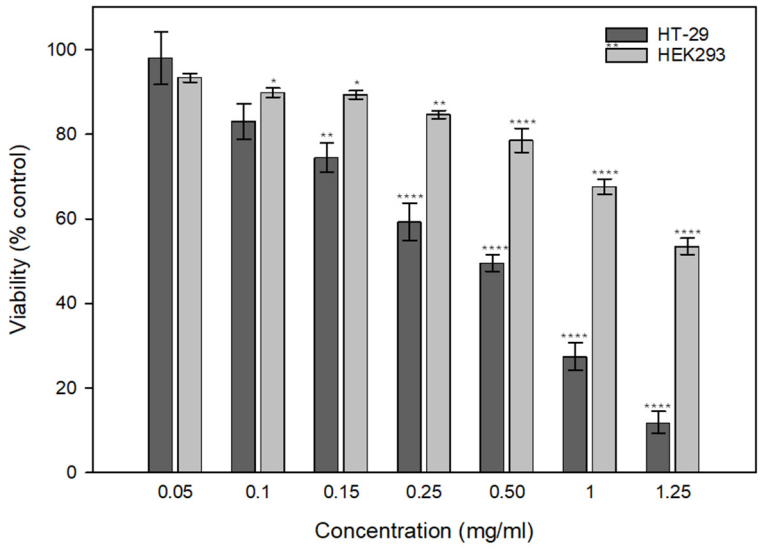

Figure 2. Cell viability of HT-29 and HEK293 cells treated with different concentrations of *B. bifidum* in 24 h. All data shown represent the mean of at least two independent experiments ± SEM. * shows the statistically significance of data achieved by One-Way ANOVA (*p < 0.05). Significance was relative to the control. Bars represent fold differences of mean normalized % viability values ± SEM (n=4).

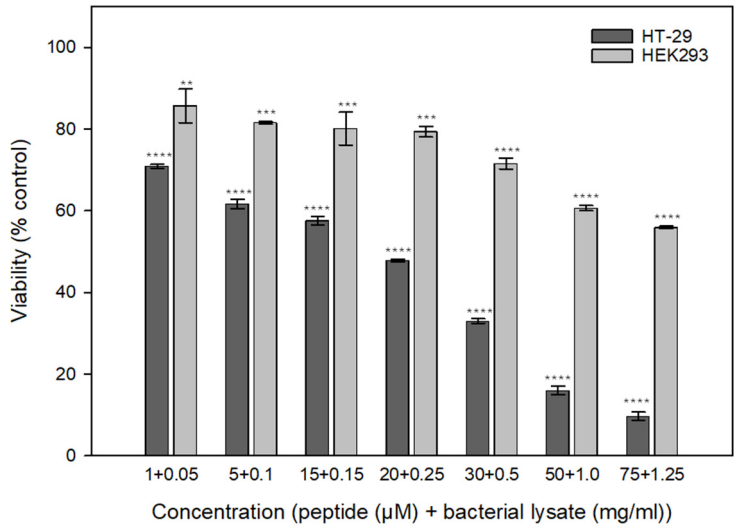

Figure 3. Cell viability of HT-29 and HEK293 cells treated with different concentrations of D-peptide B and *B. bifidum* in 24 h. All data shown represent the mean of at least two independent experiments ± SEM. * shows the statistically significance of data achieved by One-Way ANOVA (*p < 0.05). Significance was relative to control. Bars represent fold differences of mean normalized % viability values ± SEM (n=4).

combination of peptide (20 µM) and bacterial lysate (0.25 mg/ml) indicated the desired IC50.

2. RT-qPCR and gene expression

To evaluate the effect of peptide, bacterial lysate, and their combination on HT-29 and HEK293 cells, the expression of *LC3A* and *CASP3* genes at their respective IC50 values was investigated. To determine the significance of changes in gene expression relative to the control group, which consisted of untreated HT-29 and HEK293 cells, statistical analysis was performed using GraphPad Prism 8 software. Peptide-treated HT-29 cells showed an estimated a 5.5-fold increase in *LC3A* gene expression, and a 3.5-fold rise in *CASP3* expression in comparison to the control. In HT-29 cells treated with bacterial lysate, the expression of *LC3A* gene increased by approximately 4-fold, and the expression of *CASP3* gene increased by almost 2-fold

compared to the control. When HT-29 cells were treated with a combination of peptide, and bacterial lysate, the expression of *LC3A* gene increased by a factor of 7, and the expression of *CASP3* gene increased by approximately 7.5-fold. In HEK293 cells treated with peptide, bacterial lysate and their combination, the expression of *LC3A* and *CASP3* genes showed no statistically significant expression ($p > 0.9999$) (Figure 4). In contrast, in HT-29, as anticipated, the genes showed a significant increase in treated cells by: bacterial lysate, ***$p = 0.0009$; peptide, ****$p < 0.0001$; combination of peptide and bacterial lysate, ****$p < 0.0001$ for *CAS3* gene; and bacterial lysate, ***$p = 0.0003$; peptide, ****$p < 0.0001$, and combination of peptides and bacterial lysate, ****$p < 0.0001$ for *LC3A* gene.

Tukey's test is a statistical test used to identify significant differences among multiple groups in an experiment. Comparing group means and identifying which groups vary considerably from one another is a typical usage for GraphPad Prism. As an extra investigation on the significance of the findings, Tukey's

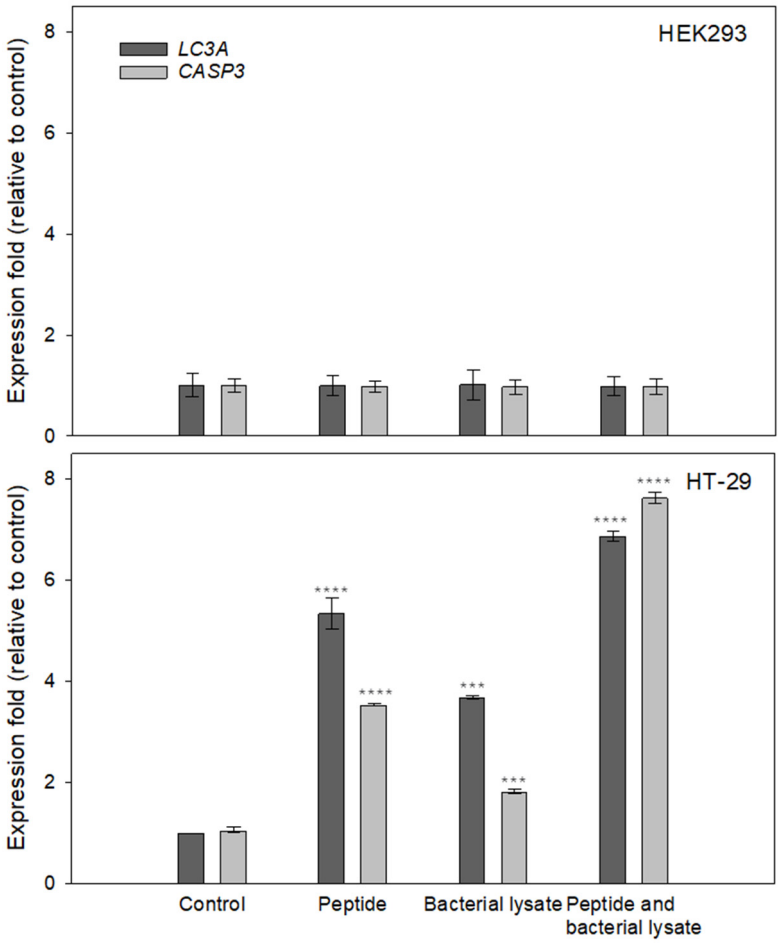

Figure 4. Expression of *LC3A* and *CASP3* genes in HT-29 and HEK293 cells. Peptide, bacterial lysate, and a combination of peptide, and bacterial lysate enhanced *LC3A* and *CASP3* genes expression in HT-29 cells. HT-29 and HEK293 cells were exposed to IC50 doses of each substance obtained by the MTT assay. Gene expression was assessed via RT-qPCR. Fold changes were normalized to *GAPDH* using comparative Ct method which are relative to the gene expression in untreated cells. The expression of these genes in HEK293 cells had no statistically significant changes ($p > 0.9999$) vs the respective untreated control at a given time point.
In HT-29 cells the genes expression changes were as follows: bacterial lysate, ***$p = 0.0009$; peptide ****$p < 0.0001$; combination of peptides and bacterial lysate, ****$p < 0.0001$ for *CAS3* gene; and bacterial lysate, ***$p = 0.0003$; peptide, ****$p < 0.0001$; and combination of peptides and bacterial lysate, ****$p < 0.0001$ for *LC3A* gene to corresponding control *$p < 0.05$.

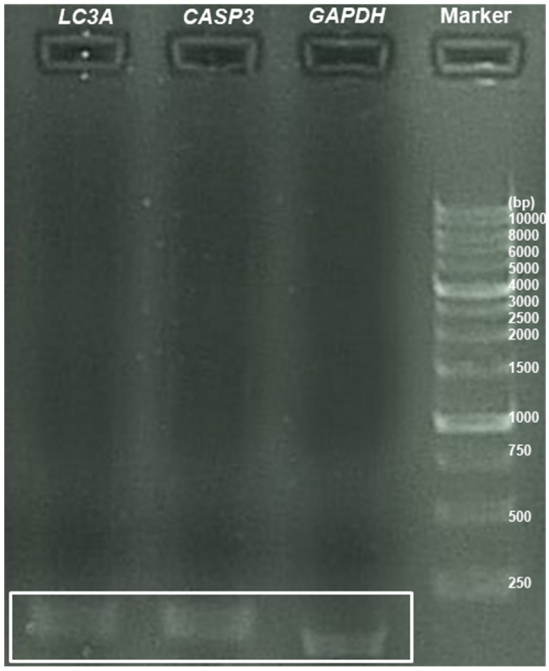

Figure 5. PCR products on a 2% agarose gel. Three distinct bands centered around 200 bp, aligning with the expected lengths of the amplified products as per NCBI Primer-BLAST data

test was used to evaluate the gene expression in each treated group in relation to the other treated groups (Appendix B). When HT-29 cells were treated with peptide as opposed to bacterial lysate, there was a substantial increase in the expression of *LC3A* gene (**p = 0.0019). When HT-29 cells were treated with both peptide and bacterial lysate, *LC3A* gene expression significantly increased as compared to when HT-29 cells were treated with peptide alone (***p = 0.0026) and bacterial lysate (***p = 0.0002). When HT-29 cells were treated with peptide as opposed to bacterial lysate, there was a substantial increase in *CASP3* gene expression (****p < 0.0001). Furthermore, HT-29 cells treated with peptide and bacterial lysate demonstrated a substantial increase in *CASP3* gene expression when compared to HT-29 cells treated with peptide (****p < 0.0001) and bacterial lysate (****p < 0.0001).

3. Amplified products on the agarose gel

The amplified product fragments were observed on the agarose gel, revealing no evidence of nonspecific products (Figure 5). The resulting gel showed three distinct bands. Despite the absence of markers for sizes smaller than 250 bp on the ladder, it was evident that the distinct bands were centered around 200 bp, aligning with the expected lengths of amplified products as per NCBI Primer-BLAST data: *LC3A* gene (200 bp), *CASP3* gene (162 bp), and *GAPDH* (200 bp). This step validated the amplification of specific gene fragments.

Discussion

Understanding the effect of different treatments on gene expression in cancer cells is critical for determining their therapeutic potential. The present research looked at the combined impact of D-peptide B and *B. bifidum* lysate on *LC3A* and *CASP3* gene expression in HT-29 cells. The area of cancer therapy has seen an increase interest in investigating complementary alternative therapeutic techniques in recent years. Among these approaches, AMPs and bacteria have emerged as promising candidates for cancer treatments. To date, the effectiveness of AMPs and bacteria on various cancer cells has been widely studied (19, 20, 21). Researchers considered these compounds promising candidates as complementary strategies for treating cancers. AMP drugs do not generally have side effects and should not cause problems for the patient in terms of their low toxicity (22). Bacteria are beneficial in the treatment of CRC due to their demonstrated anticancer properties as well as their role in maintaining a balanced gut microflora.

Induced cell death is one of the most common approaches to remove cancer cells (23). Peptides, based on Maraming et al. (2018), may decrease the development of human colon cancers by causing caspase-independent apoptosis (24). In another study, Li et al. (2019) shown *in vitro* that peptides reduced the number of viable CRC cells by apoptosis and elevated the expression of pro-apoptotic proteins in the cytoplasm surrounding the nucleus (25). Our study concentrated on apoptosis-dependent gene expression, and we discovered that it rose in HT-29 cells that had received treatment. Consistent with the current study's findings, Saleh et al. (2022) investigated the anticancer effects of a conjugated peptide against the HT-29 cell line, which led to a significant increase in *CASP3* gene expression and induced cell death via apoptosis and necrosis processes. Bacteria, in the form of probiotics, living oral microbial supplements, or bacterial lysate benefit gut health in terms of the establishment of microbial balance, and nutritional balance in the human intestine (26). Furthermore, bacteria, such as *Lactobacilli* and *Bifidobacteria*, have shown to have anticancer properties, generate antioxidative enzymes, are authoritative to responsive oxygen species, and neutralize diverse carcinogens. Furthermore, bacteria can control the cell cycle in cancer cells by repressing their multiplication and making them susceptible to apoptosis (27, 28). The benefits of bacteria are not

restricted to the anticipation of intestinal cancers, but they can incorporate the avoidance of symptoms, and complications in patients experiencing colorectal surgery for cancer and who receive intestinal cancer treatment (29).

To the best of the authors' knowledge, the current work is the first research to look into the combined effects of D-peptide B and *B. bifidum* on HT-29 cell line. The goal of the current study was to examine the possible anticancer activities of D-peptide and *B. bifidum* on the HT-29 cell line. Studies have shown that several cancer cell types, including CRC, are resistant to apoptosis. But *B. bifidum* could be able to get past this resistance by first triggering pro-caspases, which would allow caspases to break down the barrier. The researchers discovered that in human CRC, D-peptide B and *B. bifidum*, both alone and together, might trigger apoptosis, and autophagic pathways. Additionally, in the HT-29 cell line, peptides, bacterial lysate alone, and bacterial lysate in combination triggered the greatest and lowest amounts of autophagy and death. The selectivity of D-peptide B for cancer cells was further shown by Iwasaki et al. (2009), who reported that it exhibited no cytotoxicity against normal rat leukocyte cells but instead hindered the proliferation of many cancer cell lines. Since the mechanism of action of the substance was not specified in their analysis, research is necessary to establish the most practicable component or compounds and the role of D-peptide B as a preventive or therapeutic agent in CRC. So far, *B. bifidum* strains were used to treat **CRC (30, 31, 32, 33).** Saber et al. (2017) claimed that *B. bifidum* inhibited the growth of a few cancer cell lines, including HT-29 (30), and the authors of the current study discovered that *B. bifidum* might induce apoptosis and autophagy in the HT-29 cell line by upregulating *LC3A* and *CASP3* gene expression. Furthermore, our findings suggested that combined effects of D-peptide B and *B. bifidum* could increase the expression level of *CASP3* apoptotic gene, and *LC3A* autophagic gene in the HT-29 cell line, suggesting its potential in treating CRC.

Peptides have been used as medications for decades, their use, however, may be restricted due to a number of variables, including their high flexibility, slow rate of drug transfer from membranes, and breakdown by enzymes such as proteases. *CASP3* gene is important in the apoptotic pathway, which is essential for cancer cell elimination and tissue homeostasis. D-peptide B treatment significantly increased *CASP3* gene expression in HT-29 cells, based on our results. This upregulation suggests D-peptide B may induce apoptotic pathways, potentially promoting increased cancer cell death. These results align with previous studies that showed pro-apoptotic properties of D-peptide B in various cancer cell lines. In addition to *CASP3*, we examined the impact of *B. bifidum* lysate on gene expression in HT-29 cells. *B. bifidum* is a probiotic strain known for its immunomodulatory effects and potential anticancer properties. Moreover, our results showed a significant upregulation of *LC3A* gene following treatment with *B. bifidum* lysate. The *LC3A* gene is linked to autophagy, a cellular mechanism that degrades, and recycles damaged cellular components. Since HT-29 cells trigger *LC3A*, it is likely that *B. bifidum* activates autophagy, which might aid in the removal of cancer cells or make them more susceptible to other anticancer treatments.

The focus of the current study was to investigate the combined effect of D-peptide B and *B. bifidum* lysate on gene expression in HT-29 cells. The combination treatment resulted in a potential synergistic upregulation of *LC3A* and *CASP3* genes compared to individual treatments, evidenced by the results of gene expression. This suggests a potential cooperative effect of D-peptide B and *B. bifidum* lysate in promoting apoptosis, and autophagy in HT-29. The synergistic upregulation of *LC3A* and *CASP3* genes indicates that the combination treatment may have a greater effect on cancer cell death and cellular clearance mechanisms. The results showed the potential of D-peptide B and *B. bifidum* lysate as individual treatments to modulate *CASP3*-mediated apoptosis and *LC3A*-mediated autophagy in HT-29 cells. However, the synergistic effects observed with the combination treatment requires further studies to elucidate the underlying mechanisms driving this enhanced response. To have a thorough knowledge of their impact on cancer cell viability and therapeutic success, further research should look at the downstream consequences of gene expression changes brought about by these therapies, such as evaluating apoptotic and autophagic markers. In general, this research offers significant understanding of how D-peptide B, *B. bifidum* lysate, and their combination affect *LC3A* and *CASP3* gene expression in HT-29 cells. The results imply that these substances may alter the pathways involved in autophagy, and apoptosis, which are essential for the survival and elimination of CRC cells. The authors reiterate the need for additional research to validate the therapeutic implications of targeting *LC3A* and *CASP3* gene expression in complementary therapy for CRC.

Conclusion

Advancement in cancer research has led to a shift in focus towards innovative approaches that can complement conventional treatments and enhance

patient outcomes. At certain concentrations, AMPs like D-peptide B and bacteria like *B. bifidum* show promise as natural chemicals with anticancer activity that may be used as a complementary therapy for CRC without harming healthy cells. The current research examined the anticancer properties of D-peptide B and *B. bifidum* against HT-29 cells. The findings showed a synergistic impact between D-peptide *B* and *Bifidum* lysate led to a potential higher upregulation of the expression of *LC3A* and *CASP3* genes via the possible induction of autophagic and apoptotic pathways. This implies that the combination of these two compounds could be a potential complementary treatment for CRC, and further research is mandatory to explore this possibility.

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Appendix A. The precise quantities of materials employed in the RT-qPCR methodology

Sample	*GAPDH			*LC3A			*CASP3			Final volume (µl)
	Master	H_2O	Primer F and R	Master	H_2O	Primer F and R	Master	H_2O	Primer F and R	
Peptide	10	7	2	10	7	2	10	7	2	20
Bacterial lysate	10	7	2	10	7	2	10	7	2	20
Peptide and bacterial lysate	10	7	2	10	7	2	10	7	2	20
Control	10	7	2	10	7	2	10	7	2	20

* 1 µl of each gene sample was utilized.

Appendix B. The results of the statistical analysis of the Tukey's test

Sample	Mean Diff.	95.0% CI of diff.	Significance	Adjusted p-value
Comparison of each treated group with other treated group in terms of the difference in *LC3A* gene expression in HEK293 cells				
Peptide vs bacterial lysate	0.007000	-0.5538 - 0.5678	None	> 0.9999
Bacterial lysate and peptide vs bacterial lysate	0.01800	-0.5428 - 0.5788	None	= 0.9991
Bacterial lysate and peptide vs peptide	0.01100	-0.5498 - 0.5718	None	= 0.9998
Comparison of each treated group with other treated group in terms of the difference in *CASP3* gene expression in HEK293 cells				
Peptide vs bacterial lysate	-0.01000	-0.9581 - 0.9381	None	> 0.9999
Bacterial lysate and peptide vs bacterial lysate	-0.01000	-0.9581 - 0.9381	None	> 0.9999
Bacterial lysate and peptide vs peptide	0.000	-0.9481 - 0.9481	None	> 0.9999
Comparison of each treated group with other treated group in terms of the difference in *LC3A* gene expression in HT-29 cells				
Peptide vs bacterial lysate	-1.663	-2.338 - 0.9880	**	= 0.0019
Bacterial lysate and peptide vs bacterial lysate	-3.199	-3.874 - 2.524	***	= 0.0002
Bacterial lysate and peptide vs peptide	-1.536	-2.211 - 0.8610	**	= 0.0026
Comparison of each treated group with other treated group in terms of the difference in *CASP3* gene expression in HT-29 cells				
Peptide vs bacterial lysate	-1.710	-2.273 - 1.147	***	= 0.0009
Bacterial lysate and peptide vs bacterial lysate	-5.800	-6.363 - 5.237	****	< 0.0001
Bacterial lysate and peptide vs peptide	-4.090	-4.653 - 3.527	****	< 0.0001
Comparison of each treated group with other treated group in terms of the difference in *LC3A* gene expression in HEK293 cells				
Peptide vs bacterial lysate	0.007000	-0.5538 - 0.5678	None	> 0.9999
Bacterial lysate and peptide vs bacteria	0.01800	-0.5428 - 0.5788	None	= 0.9991
Bacterial lysate and peptide vs peptide	0.01100	-0.5498 - 0.5718	None	= 0.9998
Comparison of each treated group with other treated group in terms of the difference in *CASP3* gene expression in HEK293 cells				
Peptide vs bacterial lysate	-0.0100	-0.9581 - 0.9381	None	> 0.9999
Bacterial lysate and peptide vs bacteria	-0.0100	-0.9581 - 0.9381	None	> 0.9999
Bacterial lysate and peptide vs peptide	0.0000	-0.9481 - 0.9481	None	> 0.9999
Comparison of each treated group with other treated group in terms of the difference in *LC3A* gene expression in HT-29 cells				
Peptide vs bacterial lysate	-1.6630	-2.3380 - 0.9880	**	= 0.0019
Bacterial lysate and peptide vs bacteria	-3.1990	-3.8740 - 2.5240	***	= 0.0002
Bacterial lysate and peptide vs peptide	-1.5360	-2.2110 - 0.8610	**	= 0.0026
Comparison of each treated group with other treated group in terms of the difference in *CASP3* gene expression in HT-29 cells				
Peptide vs bacterial lysate	-1.7100	-2.2730 - 1.1470	***	= 0.0009
Bacterial lysate and peptide vs bacterial lysate	-5.8000	-6.3630 - 5.2370	****	< 0.0001
Bacterial lysate and peptide vs peptide	-4.0900	-4.6530 - 3.5270	****	< 0.0001