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Anticancer effect of postbiotic derived from fermented milk of *Lactobacillus helveticus* MTCC 5463 on HT-29

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There is increasing interest in postbiotics as potential therapeutic agents due to their biofunctional properties. As non-viable bacterial products or metabolites that offer health benefits, their anticancer potential warrants investigation. This study focuses on examining the effects of postbiotics derived from lactic acid bacteria fermented milk, specifically from *Lactobacillus helveticus* MTCC 5463, on colon cancer cells. This study screened postbiotics (cell-free supernatants) derived from the fermented milk of four LAB strains for various biofunctional activities. The most promising postbiotic, derived from *Lactobacillus helveticus* MTCC 5463 was then assessed for its anticancer effects on the HT-29 colon cancer cell line using the MTT assay. The study used the RT-PCR method to examine the impact of the postbiotic on the expression levels of genes associated with apoptosis, including *Bax, Bcl-2*, *Caspase-8*, *cyclin D1*, and *p53*. After 24, 48, and 72 h of treatment, the IC50 values of postbiotic were 3.0, 1.5, and 1.0 mg/mL, respectively. Gene expression analysis via RT-PCR revealed upregulation of pro-apoptotic genes (*Bax* and *Caspase-8*) and downregulation of antiapoptotic genes (*Bcl-2*, *cyclin D1*, and *p53*) in HT-29 cells treated with the postbiotic compared to untreated control cells. These findings suggest that the postbiotic derived from the fermented milk of probiotic MTCC 5463 could serve as a promising biological agent against colon cancer.

Keywords: Cell-free supernatant, Colon cancer, Lactic acid bacteria, Gene expression, Apoptotic

Colon cancer (CC) stands as the third most prevalent cause of cancer-related fatalities globally for both men and women. Nutrition is considered the primary factor contributing to about 70% of colon cancer cases, with dietary habits representing significant risk elements for the illness[1,2]. While anticancer medications are utilized to varying extents in combating CC, they often come with high expenses and adverse effects, including the destruction of healthy cells and the development of drug resistance in cancerous cells. As a result, there is an increasing interest among researchers in exploring natural foods and their components for their potential in preventing and treating cancer.

The fermentation of milk by Lactic acid bacteria (LAB) yields diverse array of metabolites with beneficial health effects. There has been a noticeable increase in research interest surrounding these metabolites, referred to as postbiotics, and their probiotic advantages for human health in recent years[3-6]. Postbiotics encompass bacteriocins, soluble or secreted factors, and cell-free supernatants. Researchers have identified several potential anticancer mechanisms of postbiotics derived from probiotic strains. These include antiproliferative activity against cancer cell lines[8,9]; induction of apoptosis in cancer cells[7,10] and cytoprotective effects attributed to organic acids, bioactive peptides, and various cell surface components produced during fermentation[10]. Since the metabolites produced during fermentation are influenced by both the fermentation substrate and the metabolic characteristics of the strain used, the health benefits of postbiotics are substrate and strain specific. Thus, it is essential to assess the potential anticancer effects of postbiotics from specific strains and substrates.

Lactobacillus helveticus is commonly found in fermented foods and specific strain is utilized as a probiotic. Milk fermented with *L. helveticus* R389 has been found to delay breast tumor growth by reducing interleukin-6 (IL-6) levels and increasing IL-10 levels in serum, mammary glands, and tumor-infiltrating immune cells. The MTCC 5463 strain of *L. helveticus* was originally isolated from the vaginal tract of a

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healthy adult female in India. This strain has demonstrated the ability to grow in the presence of 0.3% sodium taurocholate, deconjugate bile acids, and reduce cholesterol levels *in vitro*. Additionally, it exhibits significant antimicrobial activity against various pathogens such as *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica serovar Typhi*, and *Escherichia coli*. The strain produces extracellular polysaccharides and can adhere to cells of the human carcinoma cell line HT-29. Studies have reported a hypocholesterolemic effect of *L. helveticus* MTCC 5463 in human subjects with different cholesterol levels, as well as positive immunomodulating effects in a chick model[11]. However, the potential anticancer activity of fermented milk postbiotic of *L. helveticus* MTCC 5463 against colon cancer remains to be explored.

This study aimed to investigate the anticancer effect of the postbiotic derived from the fermented milk of the probiotic strain *Lactobacillus helveticus* MTCC 5463 on the HT-29 colon cancer cell line. The study also explored the effect of the postbiotic on the expression levels of anti- or pro-apoptotic genes, including *Bax*, *Bcl-2*, *Caspase-8*, *cyclin D1*, and *p53*, to elucidate its mode of action. The findings of this research will contribute to our understanding of this topic and provide consumers with valuable insights to make more informed choices regarding the health benefits of fermented milk products.

Materials and Methods

Strains and culture conditions

The strains *Streptococcus thermophilus* MTCC 5460 (MD2), *Lactobacillus helveticus* MTCC 5463 (V3), *Lacticaseibacillus rhamnosus* MTCC 5462 (I4), and *Limosilactobacillus fermentum* (BM24), as well as *Enterococcus fecalis* ATCC 29212, *Escherichia coli* MTCC 1687, and *Listeria monocytogenes* ATCC 19111, were sourced from the culture collection of the Dairy Microbiology Department, SMC College of Dairy Science, Anand, Gujarat, India. The LAB strains were activated by overnight growth in sterile reconstituted skim milk medium. Subsequently, the strains were transferred to the said medium on a weekly basis throughout the study. Other strains were activated in nutrient broth, stored at 5±2°C, and preserved in slants as well as in 80% (v/v) glycerol at -20°C.

Screening of LAB strains

Strains were screened based on the antioxidant activity, antimicrobial activity, proteolytic activity and nitroreductase activity. Antioxidant activity of the postbiotic was assessed using the ABTS (2,2-Azino-bis (3-ethylbenzothaizoline 6-sulfonic acid) assay[7]. To investigate its antimicrobial activity against *Enterococcus faecalis* ATCC 29212, *Escherichia coli* MTCC 1687 and *Listeria monocytogenes*, the well diffusion assay described by Delgado *et al.*[13]. Proteolytic activity was determined using the OPA (O-phthalaldehyde) method[14.] Method of Kang *et al*.[15] was followed for the study of nitroreductase activity.

Preparation of the postbiotic

A 12% total solids reconstituted skim milk (RSM) was prepared by adding an appropriate amount of skim milk powder to double-distilled water, followed by thorough stirring until complete dissolution. The RSM was then subjected to autoclaving. After sterilization, the milk was cooled to 40°C, inoculated with 2% v/v of active strain and then incubated at 37°C for 24 h to facilitate optimal growth. Subsequently, the samples were centrifuged at 14,000 × g at 4°C for 10 min. The resulting supernatant was filtered through a 0.22µm membrane filter. The postbiotic thus obtained was then freeze-dried and aseptically filled into sterile containers and stored at -18°C until further use.

Once the most promising strain was selected, the postbiotic derived from the fermented milk of selected strain MTCC 5463 was analysed to determine its composition (total solids, protein, lactose, and ash content) and biofunctional attributes such as α-amylase inhibition, α-glucosidase inhibition, and ACE-inhibition. Fat, total solids (TS), lactose, and ash were assessed in accordance with the method outlined by the Bureau of Indian Standards[11]. The protein content of postbiotic was determined using the macro-Kjeldahl method[17]. α-amylase inhibition, α-glucosidase inhibition and ACE-inhibition activities were measured[18]. The presence of peptides in the postbiotic was detected using a reversed-phase high-performance liquid chromatography system (Shimadzu LC-20, Kyoto, Japan) to separate distinct peptide peaks. This system utilized a binary gradient and a Thermo Fisher Scientific analytic column measuring 5µ, 250 × 4.6 mm. Sample injection was facilitated by a micro-injector (Hamilton Bonaduz AG, Switzerland) with a 20µL loop. The eluents, A and B, contained 0.01% (v/v) TFA in deionized water and in an 80:20

acetonitrile/deionized water mixture, respectively. The segregation process was carried out at a flow rate of 0.25 mL per minute at room temperature (20°C). Peptide elution times were determined[19], and a Shimadzu SPD-20A detector, configured for visible and UV wavelengths, identified a peak at 214 nm. The controls in this study comprised of unfermented milk (C1) and the drug 5-fluorouracil (5-FU).

HT-29 cell line and MTT assay

The HT-29 cell line, derived from human colonic epithelial carcinoma (colorectal adenocarcinoma), was obtained from the National Centre for Cell Science (NCCS) in Pune, India. These cells were cultured at 37°C in a 5% CO_2 environment using high-glucose Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (Hi-media, Mumbai, India). To assess the cytotoxicity of postbiotic against HT-29 cells, the MTT [3-[4,5-dimethylthiazol-2-yl]- 2,5-dipheny-ltetrazolium bromide] colourimetric method was employed. Initially, 2×10^4 cells per well were seeded into 96-well plates and left to incubate overnight in a CO_2 incubator at 37°C. Subsequently, the cells were exposed to varying concentrations of postbiotic and controls (ranging from 0.1 to 3.0 mg/mL) for 24, 48, and 72 h. Following treatment, MTT was introduced into the wells, and the reaction mixture was incubated at 37°C in a 5% CO_2 environment for 4 h. After removing the MTT dye, the formazan crystals were dissolved in dimethyl sulfoxide. Finally, absorbance was taken at 570 nm using an ELISA reader (TECAN, Switzerland) and the effect of postbiotic was expressed by IC_{50} value. The percentage survival of cells post-treatment was calculated as (OD of test/OD of control) × 100.

Gene expression study

The method outlined by Ardestani *et al.*[20] was employed to examine gene expression levels. In brief, The HT-29 cells were treated with the IC_{50} concentration of postbiotic and 5-FU drug for 24 h. Subsequently, RNA extraction and cDNA synthesis were carried out using the iScript cDNA synthesis kit (Bio-Rad). The expression levels of genes *Caspase-8*, *Bax*, *Bcl-2*, *p53*, and *cyclin D1* in HT-29 cells treated with the postbiotic and the controls for 24 h were determined using the SYBR Green method. The GAPDH gene served as an internal control. Real-time PCR reactions were performed using the Quant Studio 5 system. Primer sequences are provided in Table 1. Following the reaction, data were obtained from the device as CT (threshold cycle), and gene expression was quantified using the comparative CT method (also known as the $2^{-\Delta\Delta CT}$ method).

Statistical analysis

Data obtained were analysed using factorial completely randomized design[21]. The significance of the influence of each parameter on the specific characteristic was tested at 5.0% level of significance.

Results

Antioxidant activity

The antioxidant activity of LAB strains was assessed based on their ability to scavenge free radicals, with results presented as percentage ABTS activity (Fig. 1). Strain MTCC 5463 demonstrated significantly (P<0.05) higher activity (84.21%) compared to other strains [MD2 (82.92%), I4 (79.53%), and BM24 (76.92%)] and the control [unfermented milk (16.11%)] after 12 h of incubation. Similar trend was seen after 24 h and 36 h of incubations. Highest activity was observed after 12 h of incubation for all strains.

Antimicrobial activity

Table 2 demonstrates the antimicrobial effect of postbiotics from the strains against three pathogens. Unfermented milk served as control, showing no inhibition zones against any pathogens. Notably, the postbiotic from V3 displayed the highest antimicrobial activity against *Escherichia coli* MTCC 1687 and *Enterococcus faecalis* ATCC 29212 after 48 h of incubation. Similarly, both V3 and I4 exhibited

Table 1 — Primers used in the study

Genes	Forward primer	Reverse primer
Caspase-8	5′-GACAGAGCTTCTTCGAGACAC-3′	5′-GCTCGGGCATACAGGCAAAT-3′
Bax	5′-CCCGAGAGGTCTTTTTCCGAG-3′	5′-CCAGCCCATGATGGTTCTGAT-3′
Bcl-2	5′-GGTGCCGGTTCAGGTACTCA-3′	5′-TTGTGGCCTTCTTTGAGTTCG-3′
p53	5′-CATAGTGTGGTGGTGCCCTA-3′	5′-CACCTCAAAGCTGTTCCGTC-3′
cyclin D1	5′-GCTGCGAAGTGGAAACCATC-3′	5′-CCTCCTTCTGCACACATTTGAA-3′
GAPDH	5′-AGAAGGCTGGGGCTCATTTG-3′	5′-AGGGGCCATCCACAGTCTTC-3′

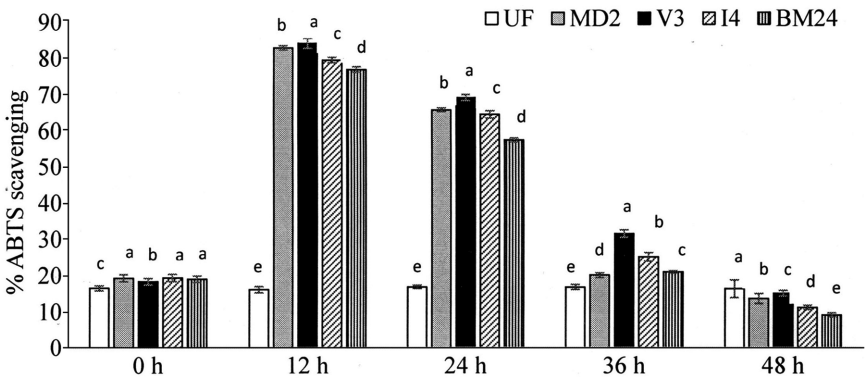

Fig. 1 — Antioxidant activity of LAB strains. [The data represent mean values and standard deviations obtained from four replicate experiments.UF = Unfermented milk, *Streptococcus thermophilus* MTCC 5460 (MD2), *Lactobacillus helveticus* MTCC 5463 (V3), *Lacticaseibacillus rhamnosus* MTCC 5462 (I4), and *Limosilactobacillus fermentum* (BM24). Different superscripts indicate significant difference among strains at specific interval of incubation at 5% level of significance]

Table 2 — Antimicrobial activity (zone of inhibition in mm) of LAB cultures

LAB strains / Fermentation Time	Pathogens		
	Listeria monocytogenes ATCC19111	*Escherichia coli* MTCC 1687	*Enterococcus faecalis* ATCC 29212
MD2 / 12 h	$0^{c} \pm 0$	$0^{b} \pm 0$	$9.50^{c} \pm 1.29$
V3 / 12 h	$11.50^{b} \pm 0.58$	$0^{b} \pm 0$	$15.25^{a} \pm 0.50$
I4 / 12 h	$12.75^{a} \pm 0.50$	$0^{b} \pm 0$	$12.25^{b} \pm 1.26$
BM24 / 12 h	$0^{c} \pm 0$	$13.00^{a} \pm 0.82$	$15.00^{a} \pm 2.16$
MD2 / 24 h	$0^{d} \pm 0$	$16.50^{a} \pm 0.58$	$13.75^{c} \pm 0.96$
V3 / 24 h	$16.75^{a} \pm 1.50$	$14.25^{c} \pm 0.96$	$14.75^{a} \pm 1.50$
I4 / 24 h	$15.50^{b} \pm 1.29$	$13.50^{d} \pm 0.58$	$14.25^{b} \pm 0.50$
BM24 / 24 h	$13.25^{c} \pm 0.96$	$15.00^{b} \pm 0.82$	$12.75^{d} \pm 0.96$
MD2 / 36 h	$0^{d} \pm 0$	$11.75^{b} \pm 0.50$	$14.00^{c} \pm 0.25$
V3 / 36 h	$17.75^{a} \pm 0.50$	$15.25^{a} \pm 0.50$	$16.00^{a} \pm 0.35$
I4 / 36 h	$17.25^{b} \pm 0.50$	$15.25^{a} \pm 0.96$	$16.00^{a} \pm 0.25$
BM24 / 36 h	$12.75^{c} \pm 0.96$	$0^{c} \pm 0$	$15.00^{b} \pm 0.82$
MD2 / 48 h	$0^{c} \pm 0$	$11.25^{c} \pm 0.50$	$15.25^{c} \pm 0.82$
V3 / 48 h	$18.25^{a} \pm 0.96$	$17.25^{a} \pm 0.58$	$17.75^{a} \pm 0.50$
I4 / 48 h	$18.25^{a} \pm 0.50$	$15.50^{b} \pm 0.50$	$16.25^{b} \pm 0.50$
BM24 / 48 h	$15.25^{b} \pm 0.96$	$0^{d} \pm 0$	$14.50^{d} \pm 0.58$

[Each observation is a mean of four replicate experiments. *Streptococcus thermophilus* MTCC 5460 (MD2), *Lactobacillus helveticus* MTCC 5463 (V3), *Lacticaseibacillus rhamnosus* MTCC 5462 (I4), and *Limosilactobacillus fermentum* (BM24). Different superscripts within a column, and within the same time interval indicate significant differences]

significant antimicrobial effects against *Listeria monocytogenes* ATCC 19111 after the same incubation period. Overall, V3 demonstrated superior antimicrobial activity against the tested pathogens.

Proteolytic activity

Fig. 2 shows the proteolytic activity of LAB strains, indicating significant differences (P<0.05) within the cultures and incubation periods. The proteolytic activity of all strains increased significantly (P<0.05) with prolonged incubation periods. The strain V3 exhibited significantly high proteolytic activity at 24 h, 36 h and 48 h of incubation.

Nitroreductase activity

The positive control *E. coli* ATCC 43888 turned the medium purple, while the LAB strains did not exhibit any colour change, indicating the lack of nitroreductase activity in these strains (Fig. 3).

α-amylase inhibition, α-glucosidase inhibition and ACE-inhibition potential of postbiotic of V3

On the basis of the results obtained for antioxidant, antimicrobial, proteolytic and nitroreductase

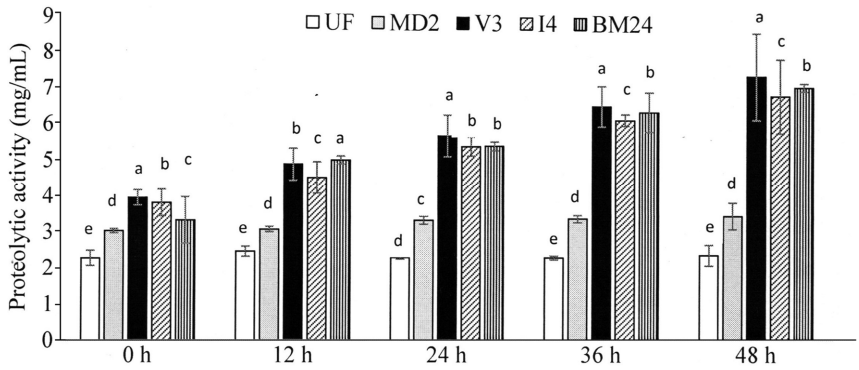

Fig. 2 — Proteolytic activity of LAB strains. [Each observation is a mean of four replicate experiments. *Streptococcus thermophilus* MTCC 5460 (MD2), *Lactobacillus helveticus* MTCC 5463 (V3), *Lacticaseibacillus rhamnosus* MTCC 5462 (I4), and *Limosilactobacillus fermentum* (BM24). Different superscripts indicate significant difference among strains at specific interval of incubation at 5% level of significance]

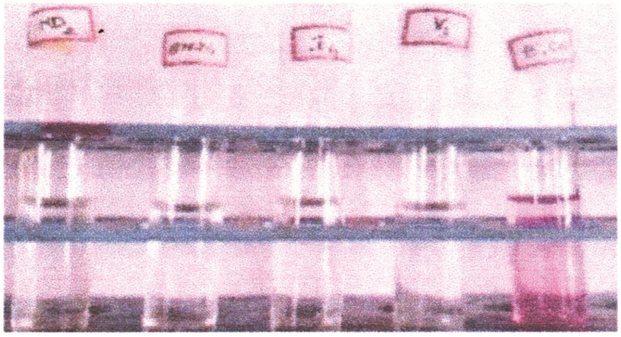

Fig. 3 — Nitroreductase activity of LAB strains. [*Streptococcus thermophilus* MTCC 5460 (MD2), *Lactobacillus helveticus* MTCC 5463 (V3), *Lacticaseibacillus rhamnosus* MTCC 5462 (I4), and *Limosilactobacillus fermentum* (BM24), control *E. coli* ATCC 43888]

activities, the strain V3 was selected for evaluation of anticancer effect on HT-29. Further, the fermented milk postbiotic of V3 exhibited α-amylase inhibition, α-glucosidase inhibition and ACE-inhibition of 69.18 %, 51.68 % and 66.19 % respectively.

Anticancer activity of postbiotic by MTT Assay

Fig. 4 depicts the antiproliferative effect of varying concentrations of postbiotic on HT-29 cells at 24, 48, and 72 h of treatment. Every concentration of postbiotic markedly decreased the survival rate of cancer cells after each individual time frame compared to the control (P<0.05). The degree of cytotoxicity was observed to be influenced by both the dosage and duration of exposure. After 24, 48, and 72 h of treatment, the IC_{50} concentrations of postbiotic were 3.0, 1.5, and 1.0 mg/mL, respectively. The IC_{50} of the 48 and 72 h treatments were significantly (P<0.05) less compared to the IC_{50} of the 24 h treatment (Fig. 5).

Gene Expression

Fig. 6 illustrates the gene expression levels in HT-29 cells following treatment with postbiotic at the IC_{50} concentration for 24 h. The proapoptotic genes *caspase-8* and *Bax* were up-regulated and the antiapoptotic genes *Bcl-2*, *cyclin D1* and *p53* were down regulated after 24 h of treatment.

Discussion

In this study, LAB strains were screened on the basis of important biofunctional activities related to potential anticancer effects such as antioxidant, antimicrobial, proteolytic and nitroreductase activity for the selection of the most promising strain. Further the fermented milk postbiotic derived from the selected strain was analysed for its proximate composition. The mean values of total solids, protein, lactose and ash content of the fermented milk postbiotic were found to be 5.99, 0.71, 4.69 and 0.61 percent respectively. Furthermore, the RP-HPLC chromatogram revealed the presence of peptides (peaks shown at 26 min to 50 min) in the postbiotic in comparison to control C1 (Fig. 7).

Peptides derived from the milk fermented by LAB have been associated with significant anticancer properties[22]. Research suggests that cytomodulatory peptides derived from milk proteins can selectively target cancer cells without affecting healthy cells[23]. Casein-derived peptides have demonstrated the ability to hinder cancer cell proliferation and promote apoptosis[24]. Specific milk peptides have been proposed to induce apoptosis in cancer cells, potentially limiting their viability[25]. Studies have shown that peptide fractions extracted from milk fermented by *L. helveticus* can effectively reduce fibrosarcoma proliferation *in vivo*[26]. The production

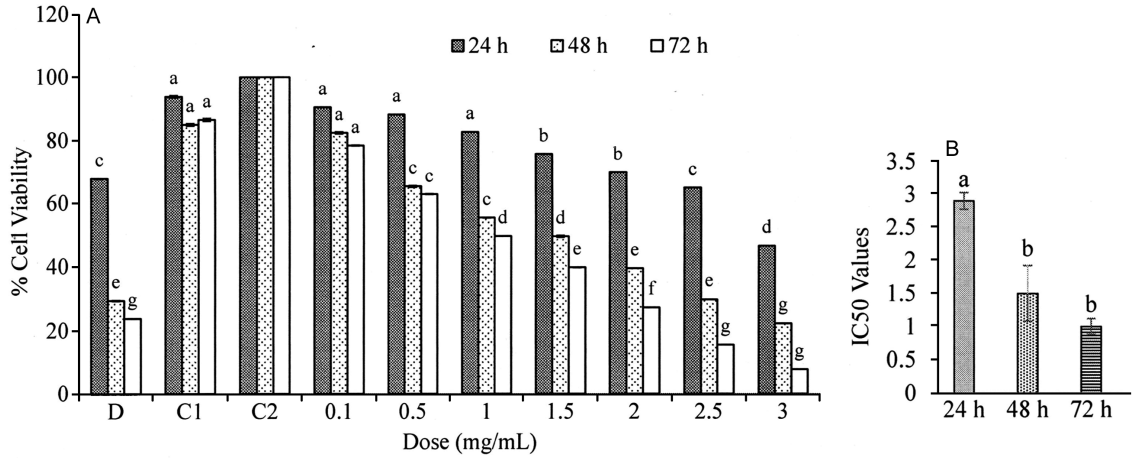

Fig. 4 — (A) Viability of HT-29 cells after 24 h, 48 h and 72 h of treatment with postbiotic measured using MTT assay.D = drug 5-FU, C1 = Unfermented milk, C2 = Untreated cells. (B) Quantitative assessment of postbiotics' efficacy against HT-29 cancer cells.

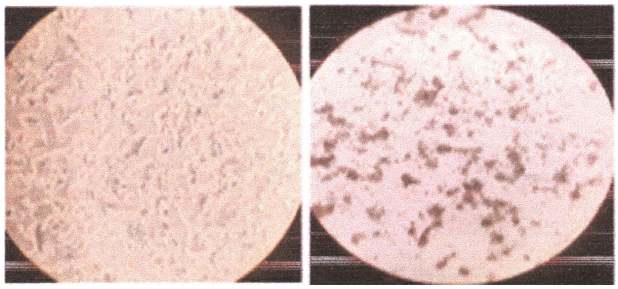

Fig. 5 — Morphology of HT-29 cells with and without the treatment under a light microscope (400× magnification)

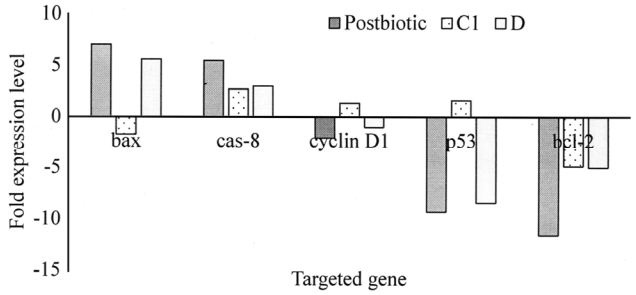

Fig. 6 — The expression levels of target genes in HT-29 cell line treated with postbiotic for 24h in comparison to controls. [C1 = Unfermented milk, D = drug 5-FU]

of bioactive peptides depends on the proteolytic enzymes of LAB and their specificity toward milk proteins, which can vary among different fermented milk microorganisms. This variability in enzymatic activity leads to the release of diverse bioactive peptides depending on the selected strains. In our study strain V3 displayed (Fig. 2) the highest proteolytic activity (7.32 mg/mL) followed by BM24 (7.01 mg/mL), I4 (6.77 mg/mL), and MD2 (3.45 mg/mL) after 48 h of incubation at 37°C in skim milk. In the study by Elfahri*et al.*[24], proteolytic activity in bovine skim milk fermented by *L. helveticus* 1315, was found to significantly increase towards the end of fermentation, with the activity being markedly strain-dependent (P<0.05). Additionally, proteolysis was observed to escalate as incubation time increased from 0 to 24 h, consistent with the findings of our study, indicating a correlation between increasing proteolytic activity and prolonged fermentation duration.

Antioxidants are known to mitigate the development of cancer by inhibiting the harm caused by free radicals to DNA, decreasing cell division prompted by oxidants, and promoting apoptosis[27,28]. Milk fermentation is reported to release bioactive peptides possessing antioxidant activity depending on the type of strain and the duration of fermentation. In this study postbiotic of strain V3 demonstrated significantly (P<0.05) higher antioxidant activity (84.21%) compared to others (Fig. 1). Similar findings were reported by Elfahri *et al.*[24] regarding the soluble extracts of fermented bovine milk by *L. helveticus* strains. Moreover, the antioxidant capacity is found to be dependent on the specific strain used[29].

The antimicrobial effect (Table 2) of postbiotics of LAB strains were studied against the pathogens *Listeria monocytogenes*, *Escherichia coli*, and *Enterococcus faecalis* owing to their potential role in colon cancer. These pathogens have been implicated in the development of colon cancer. *Enterococcus faecalis* (*E. faecalis*) is a normal inhabitant of the intestinal tract and has been frequently detected in colorectal cancer patients[30,31]. Significantly higher

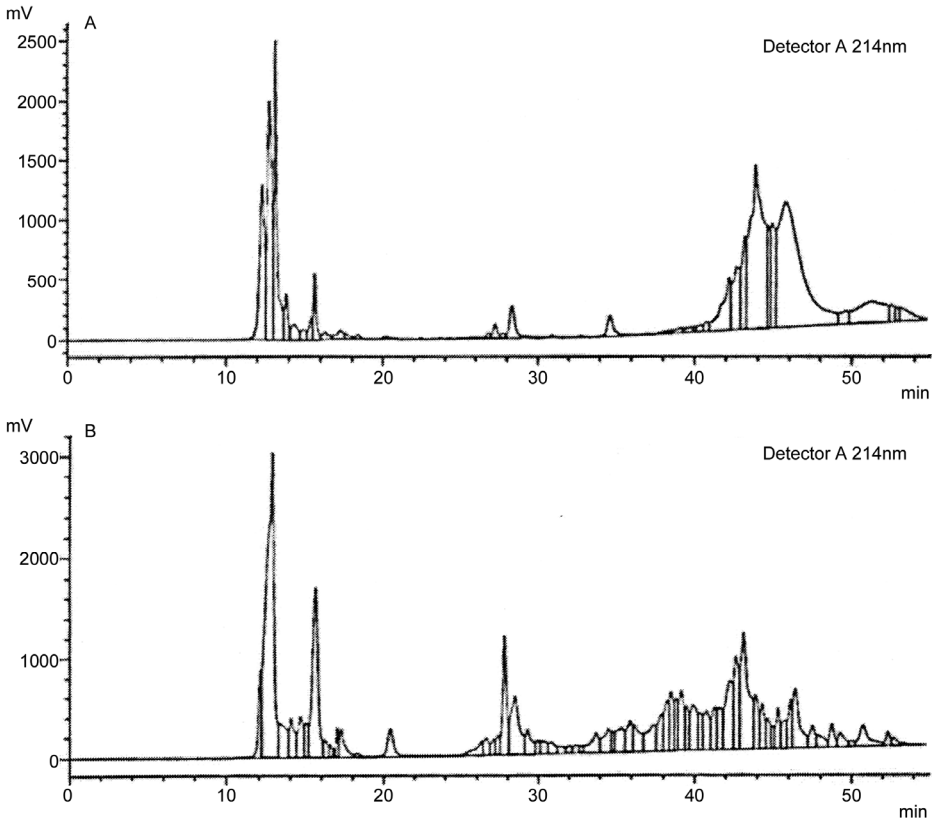

Fig. 7 — RP-HPLC chromatogram of control C1 (A) and postbiotic (B)

levels of *E. faecalis* in the feces of colorectal cancer (CRC) patients compared to healthy individuals has been reported[31]. *E. faecalis sp.* are capable of producing reactive oxygen and nitrogen species, which directly contribute to DNA damage, chromosomal instability, and point mutations[30]. The human intestine also harbors commensal bacterium *E. coli*, and studies have shown a significant association between CRC and mucosa-adherent *E. coli*[32,33]. Over 70% of mucosa samples from CRC patients contained *E. Coli*[32]. Isolates of *E. coli* from CRC patients have been found to produce colibactin, a genotoxin that disrupts the cell cycle and facilitates tumor formation by inducing mutations, genomic instability, and DNA damage in epithelial cells[33]. *Listeria monocytogenes* has also been implicated in colon cancer through the deacetylation of histone[34]. In our study, postbiotic of strain V3 showed (Table 2) relatively better antimicrobial activity against the tested pathogens. Arrioja-Breton *et al.*[35] investigated the antimicrobial potential of CFS from selected lactic acid bacteria strains against various pathogens, while Sadeghi*et et al.*[36] screened 144 lactic acid bacteria strains to identify those with the most potent antimicrobial properties. During fermentation, LAB strains generate various metabolites which can exhibit antimicrobial effects against pathogenic organisms. This antimicrobial activity may arise from the presence of organic acids, particularly lactic acid, acetic acid, propionic acid, and butyric acid, which are produced by the strains. Additionally, the decrease in *p*H, production of hydrogen peroxide, bacteriocins, and other antimicrobial substances could also contribute to this activity[37]. Moreover, LAB strains are known to produce diverse bacteriocins, which are generally effective against closely related species. Certain bacteriocins have even demonstrated a broad spectrum of activity[38].

Presence of positive nitroreductase activity in a LAB strain is associated to its cancer-causing ability. Nitroreductase transforms nitro compounds into aromatic amines which exhibit mutagenic and carcinogenic properties[39]. Positive nitroreductase activity is indicated by colour changes, typically red, pink, or purple, in the reaction medium, corresponding to the enzyme's concentration. In this study, while the positive control *E. coli* ATCC 43888 turned the media purple, our test strains did not

exhibit any colour change (Fig. 3), indicating a lack of nitroreductase activity in these cultures.

The link between diabetes and high blood pressure and the increased risk of colorectal cancer incidence has been reported[40]. A 35% elevated risk of colorectal cancer associated with high blood pressure has been reported[36]. Hence, postbiotics possess promising antidiabetic and ACE-inhibition activity that may reduce the risk of colon cancer. In our case, the postbiotic of V3 showed promising (> 50%) α-amylase inhibition, α-glucosidase inhibition and ACE-inhibition. Chen *et al.*[42] assessed the ACE-inhibitory activity of fermented milk produced with *L. helveticus* strains, revealing activity exceeding 50%.

The impact of various concentrations of postbiotic was assessed on the HT-29 cancer cell line over 24, 48, and 72 h treatment periods. As illustrated in Fig. 4(A), each postbiotic concentration notably decreased cancer cell survival rates after each time interval compared to the control (P<0.05). Notably, the administration of 3 mg/mL postbiotic at 72 h exhibited the most potent cytotoxic effect on cancer cells, resulting in the viability reduction of over 90 percent of the cells. Additionally, the concentrations of postbiotic after 24, 48, and 72 h were determined as 3.0, 1.5, and 1.0 mg/mL IC_{50}, respectively as shown in Fig. 4(B). The study findings revealed a significant decrease in the IC_{50} values for the 48 and 72 h treatments in contrast to the IC_{50} of the 24-hour treatment (Fig. 1B). Khoury *et al.*[43]investigated the cytotoxicity of kefir, a fermented milk, on HT-29 and Caco-2 cell lines. The IC_{50} of kefir for Caco-2 cells ranged from 18% at 24 h to 10% at 72 h. For HT-29 cells, the IC_{50} was achieved at 48 and 72 h, at 12% and 10%, respectively, indicating dose- and time-dependent cytotoxicity. In another study, the inhibitory effect of *Lactobacillus delbrueckii* fermentation (LBF) solution on SW620 cells was assessed using the MTT assay after 24 h. Treatment with LBF solution significantly inhibited the growth of colon cancer SW620 cells compared to the control group, with an IC_{50} value of 0.25 mg/mL, suggesting a concentration-dependent decrease in proliferation potential[44]. Elfahri*et et al.*[24] examined bovine skim milk fermented by *L. helveticus* strains for its potential in preventing colon cancer. The extract obtained after 12 h of fermentation displayed the highest anti-colon cancer activity against HT-29 cell line.

In this study, the introduction of the postbiotic visibly led to cell death, as evidenced by microscopic observations. HT-29 cells exhibited shrinkage and a change in colour, indicative of cellular demise (Fig. 5). Likely mechanisms underlying this cell death include apoptosis induction, inhibition of proliferation, and interference with the cell cycle. Further we found that the proapoptotic genes *caspase-8* and *Bax* were up-regulated and the antiapoptotic genes *Bcl-2*, *cyclin D1* and *p53* were down regulated after 24 h of treatment (Fig. 6). Postbiotics have been reported to trigger both intrinsic and extrinsic apoptosis pathways in human cancer cells by up-regulating pro-apoptotic proteins such as *Bad*, *Bax*, *caspase-3*, *caspase-8*, and *caspase-9*, while downregulating *Bcl-2*[45]. A study Ma *et al.*[46] reported that supernatants from *L. rhamnosus* GG and *B. lactis* could induce apoptosis by down-regulating *Bcl-2*, modulating *Bax*, inhibiting HT-29 cell growth, and halting cell proliferation in the G0/G1 phase. Postbiotics derived from *L. rhamnosus* GG and *L. Paracasei* IMPC2.1 have demonstrated inhibition of proliferation in human colon cancer DLD-1 and human gastric cancer HGC-27 cell lines[45]. Baghbani-Arani *et al.*[47] employed RT-qPCR to analyze gene expression in HT-29 cells following treatment with *L. acidophilus* extract for 24 h. They found that the expression levels of *Bax* and *Bcl-2* genes were higher in cells treated with 2 mg/mL cell extract of *L. acidophilus*. Similarly, Dehghani*et et al.*[48] discovered that *L. rhamnosus* supernatant up-regulated pro-apoptotic genes such as *caspase-3*, *caspase-9*, and *Bax* while down-regulating *Bcl-2* and reducing the expression levels of *cyclin D1*, *cyclin E*, and *ERBB2* genes. Another study evaluated the apoptotic effects of certain bifidobacterial species and their supernatants on colon cancer cell lines. All tested bifidobacterial supernatants significantly reduced the viability of colon cancer cells compared to the control, up-regulated *Bax* gene expression, and down-regulated *Bcl-2* expression[49].

Among the LAB, *Lactobacillus helveticus* is recognized for its high proteolytic activity, which enhances the likelihood of releasing bioactive peptides possessing various health-beneficial properties including anticancer property. Peptide fractions released from milk proteins by *L. helveticus* strain R389 demonstrated antitumor activity[50]. In our study, the fermented milk postbiotic of *Lactobacillus helveticus* MTCC 5463 is found to have promising, antioxidant, antimicrobial, and anticancer activities.

These effects of postbiotic make it a suitable therapeutic and preventive agent against colon cancer.

Conclusion

The findings indicated that the postbiotic derived from the fermented milk of the probiotic strain *L. helveticus* MTCC 5463 holds substantial promise in inhibiting cancer cell growth and inducing apoptotic cell death, particularly in HT-29 colon cancer cells. Given the varying effectiveness of probiotic bacteria against cancer based on their genus and strain, it is proposed that following thorough assessment, these indigenous bacteria could potentially be utilized as supplementary therapies for impeding cancer cell proliferation.

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Conflict of interest

The authors have no conflict of interest to declare.

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