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Original Research Article

Cytotoxic effect of paraprobiotic yogurts on cancer cell lines

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ABSTRACT

Cancer is a cellular disorder caused by changes in the mechanisms that control cell growth and differentiation. Breast cancer is the second most common cancer in women following lung cancer. Today, due to the side effects of treatment methods, many efforts have been made to discover natural compounds with a selective power in limiting cancers. This study was performed to investigate the cytotoxicity effects of paraprobiotic yogurt on breast and rectal cancer cell lines (MDA-Md, SKBR3, and SW 480). After culturing and amplifying the cancer cell line to determine the effect of supernatant toxicity, these cells were exposed to different doses of paraprobiotic supernatant, which was performed after 72 hours of MTT assay. According to the results, after adding different dilutions of paraprobiotic yogurt supernatant, YB_p-B could significantly inhibit the cell viability in three categories of cancer cells (MDA-Md, SKBR3, and SW 480) and had the greatest impact on SKBR3 ($p \leq 0.05$). The effects of cytotoxicity increased with elevating the concentration of the extract with the highest percentage of growth inhibition being related to the concentrations of 1/4, 1/3, and 1/2 ($p = 0.05$ and $p = 0.01$), respectively. Thus, the use of paraprobiotic yogurt supernatant as an effective substance in the treatment of cancer is recommended. Accordingly, future research could explore its substances to be potentially used in the treatment of cancer.

1. Introduction

Today, the use of probiotic bacteria has become a health-maintaining activity. However, there are limitations to their use. The first group of problems is related to the nature of their viability. Prescribing live probiotic bacteria to people of all ages and physical conditions does not always have the same positive effects. Live bacteria play a very opposite role in patients with weakened immune systems and can be dangerous. Among these cases, we can refer to people with Crohn's disease. Alternatively, the opposite effect of probiotics has also been observed in the elderly and infants who have not yet developed an immune system. Other issues raised by live probiotics include antibiotic resistance and the possibility of transfer of resistance genes to pathogenic bacteria entering the human intestine.[1] As there are pathogenic bacteria in the human gut microbiome, acquiring antibiotic resistance in these bacteria can be problematic. There is evidence that bacteria involved in food production can also be a source of antibiotic resistance.

Today, many probiotic products containing Lactobacillus acidophilus and Bifidobacterium bifidum are produced.[2] The selection of probiotic bacteria requires some criteria the most important of which being associated with GRAS group, whose beneficial effects on the health of living organisms have been proven (in and out of the body). This group is resistant to gastric juice and its acidity, salts, bile salts and digestive enzymes, having the desired ability to adhere to epithelial cells, having high retention activity or biological resistance against harmful bacteria,

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resistance to antibiotics and phage bacteria, have the desired technological properties in the production of microbial cultures (in the form of freeze-dried or lyophilized, severe freezing or spray-dried) as well as food and pharmaceutical products.[3] Reported that drying of Lactobacillus ruteri LR6 cells with sorbitol or xylitol coated with xylitol and drying of the fluidized bed with various excipients, which L. reuteri cells coated with xylitol and dried without support or with milk powder without Fats are better protected as excipients, and milk solids as excipients and drying of the fluidized bed help maintain high cell viability. Bifidobacteria are known to be an important class of probiotics that are inherently resistant to a range of antibiotics such as streptomycin. The second important problem is related to the process of production and maintenance of living and vital bacteria. Most of the bacteria used belong to the Lactobacillaceae family, which are non-spores and in adverse environmental conditions, they get damaged and lose their efficiency over time and during maintenance. On the other hand, the possibility of providing a cold chain along the transportation route often requires high costs. One method is use of certain foods such as dairy products or beverages as a probiotic carrier. It is also common to apply different methods to stabilize these bacteria to maintain cell survival. However, the interaction of living microorganisms and the environment can also cause problems for this solution. Accordingly, various solutions have been proposed in recent years to solve the aforementioned problems, the most effective of which is the use of paraprobiotics. The definition provided by FAO and the World Health Organization for probiotics is limited to living microorganisms. However, it has been reported that inanimate (dead), non-cultured, and potentially immune-activated microbial cells also provide health benefits to hosts.[4] In addition, the terms paraprobiotics and postbiotics have been proposed to define non-viable bacterial products or metabolic by-products of probiotic microorganisms with biological activity and bioactivities in the host.[5] However, in cases where non-viable bacterial structures can have a biological activity in the host, paraprobiotics may be known as postbiotics.[6] In the relevant literature, the term paraprobiotic is a combination of "para" and "probiotic" as "next to life", which is not convincing. In addition, given its literal meaning of "para" (in side) in relation to "probiotics", it may be understood that paraprobiotics can have their activity whose effectiveness and activity depend only on the probiotics and the presence of probiotics. With the above in mind, the term paraprobiotic is somewhat confusing. Note that the words "inactive probiotic", "dead probiotic", and "inanimate probiotic" are also used as synonyms for paraprobiotic. Paraprobiotics are whole or broken cells of probiotics or crude cell extracts that, when used orally or topically in the right dose, will have a beneficial effect on humans or animals.[7] The benefits of using paraprobiotics and postbiotics as functional compounds in dairy products as well as the analysis of analytical methods with the potential to be used in quality control of these products have been discussed.[6] Dairy products supplemented with paraprobiotics and postbiotics are simpler and easier in terms of industrial transportation and commercialization than probiotic products. They have little or no interaction with matrix compounds or food ingredients thereby enhancing the shelf life. They remain stable in a wide range of PHs and temperatures, allowing food / materials with higher acidity to be added before heat treatment, so that their performance is not compromised.[8] Paraprobiotics can be found in probiotic formulas at concentrations ranging from 1×10^5 to 1×10^{14} bacteria per unit dose (ml, gram, tablet or capsule). Various methods for inactivation of microorganisms have been used to produce paraprobiotics. The heat process is the most common method for inactivating microorganisms. Among these, pasteurization and sterilization are more important.[6,9] The effect of heat treatment on microorganisms depends on several factors such as cell type, vegetative or spore form, physical and chemical conditions of the culture medium, growth stage, water activity, and heating method. Other methods for inactivation include ultrasound, high pressure, ultraviolet, ionizing radiation, electric field heating, ohmic, supercritical CO2, dehydration, and substrate pH change.[9] The positive effects of paraprobiotics on health include inhibiting pathogens, modulating intestinal microbes, improving intestinal injuries, treating diarrhea, modulating inflammation, reducing lactose intolerance, lowering cholesterol, mitigating respiratory disease, improving liver diseases caused by alcohol consumption, preventing cancer progression, treating atopic dermatitis, inhibiting tooth decay, and treating colitis.[7]

Recent research has shown that cancer is currently one of the leading causes of death in the world. Breast cancer is the second most common cancer in women following lung cancer.[10] This cancer is responsible for 33% of all women's cancers and 20% of cancer mortality. The prevalence of breast cancer in developing countries is growing and has become the most common malignant disease in many parts of the world. Colorectal cancers are cancers that can be prevented with screening programs as well as monitoring and prevention with chemotherapy (nutritional or pharmacological treatments).[11] In this regard, both paraprobiotics L. Paracase IMPC2 and Rhamnosus GG showed anti-proliferative activity and anti-apoptotic effects in gastric cancer cell lines (HGC-27 and colorectal DLD-1) in Dolbeku (DMEM) and RPMI (108 cfu / ml) media. Different paraprobiotic sections of Lactobacillus such as whole cells, thermally inactivated cells, cell wall, peptidoglycan, and cytoplasmic sections can show antiproliferative effects against human cancer cell lines.[12]

This study explored the effect of paraprobiotic yogurt on cancer cells (breast) (L. acidophilus and B. lactis). If it is effective, it can be used in the treatment of this type of cancer and other cancers.

2. Materials and Methods

2.1. Bacteria culture

Two traditional probiotic bacteria Bifidobacterium animalis subsp. lactis BB-12 and Lactobacillus acidophilus ATCCSD 5221 were provided by Zist takhmir (Tehran, Iran). The direct-in vat-set (DVS) pouches of commercial yogurt starter culture (containing Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus) were supplied by Chr-Hansen (Horsholm, Denmark). Skim milk powder was provided by Pegah Milk Industries. Probiotics and starter cultures were kept at -18°C until utilized.

2.2. Cell culture

In this study, three categories of cancer cells purchased from Pasteur Institute of Iran were used for in vitro culture, which included SK-BR-3 cell lines (Human-Breast). This cell line was derived from a pleural effusion. The initial cultivation was in EMEM with 10%FBS and more recently McCoys 5a with 10-15% FBS, where the culture Medium was McCoys 5a + 10% FBS. The cell line was adapted to RPMI + 10% FBS in NCBI, with the preservation medium being FBS + 10% DMSO.

MDA-MB-231 cell lines (Human-Breast) and Culture Medium: The base medium for this cell line is ATCC-formulated Leibovitz's L-15 medium and fetal bovine serum to a final concentration of 10%. The cell line was adapted to RPMI + 10% FBS in NCBI. Preservation Medium: FBS+ 5% DMSO. Next were SW 480 cell lines (Human-Colon) whose culture medium was Leibovitz's L-15 medium + 10% FBS. The cell line was adapted to RPMI 1640 + 10% FBS in NCBI where the preservation medium was FBS + 10% DMSO. Finally, the cells were incubated at 37° C with 90% humidity and 5% CO2.

2.3. Cells treatments (Investigation of cell viability)

(MTT) test is one of the methods that is used to evaluate the toxic effects of various substances on different cell lines, including cancer and non-cancer. The cell viability was evaluated by (MTT) test (3,5,5-dimethylthiazole 2-yl 2,5-diphenyltetrazolium). The (MTT) added to the culture medium changes to formazan dye through the dehydrogenase activity of living cells. As the dehydrogenase content of cells of a type is relatively constant, the amount of formazan produced is proportional to the number of cells. In summary, the cells were placed in 96 wells of cellular platelets and at 72 hours after adding the extract to the cells, the supernatant was discarded with the cells reconstituted with 0.5 mg/ml (MTT) solution in PBS at 4°C. Using 100 μl of Dulbecco's Modified Eagle's Medium (DMEM), the absorption of light at 750 nm was read by an ELISA reader. There is a direct relationship between the amount of absorption (color intensity) or the number of living cells.[13]

2.4. Preparation of paraprobiotics

Freeze-dried and pure cultures of B. animalis subsp. lactis BB-12 and L. acidophilus ATCCSD 5221 were provided by Zist Takhmir Company (Tehran Iran) and stored at -18 řC. For preparing heat-killed cells, these organisms were killed through autoclaving at 121°C for 15 min[9,14] at a concentration of 107 cfu/L in distilled water (20 mL), after which they were cooled in an ice bath. The viable count of the heat-killed bacteria (MRS agar plate, 48 h, 37°C, aerobic and anaerobic incubation) was determined immediately after thermal treatment where no colonies were found after cultivation.

2.5. Paraprobiotic yogurts production

Three yogurt treatments (paraprobiotic yogurts) were produced using reconstituted skim milk powder. Reconstituted skim milk samples consisting 0.13% milk solid non-fat (MSNF) were inoculated with starter culture (L. delbrueckii subsp. bulgaricus and S. thermophilus) along with paraprobiotics (dead L. acidophilus ATCCSD 5221 or dead B. animalis subsp. lactis BB-12 or dead L. acidophilus ATCCSD 5221 + dead B. animalis subsp. lactis BB-12). The initial counts of L. acidophilus ATCCSD 5221 and B. animalis subsp. lactis BB-12 were 10^7 CFU/mL. Containers of reconstituted milk were heated at 85°C for 30 min, and then cooled in an ice bath to reach 43°C ± 0.01. The starter culture and paraprobiotics were incorporated after cooling of milk. The fermentation was performed at 42°C until pH dropped from 6.5±0.02 to 4.5±0.02. Biochemical parameters including the level of changes in pH, redox potential (RP), titratable acidity (TA), and incubation time were determined during the fermentation at 30-min intervals. When fermentation process was completed, the paraprobiotic yogurts were cooled and maintained at refrigeration temperature (4°C) for 28 days.[9]Figure 1 and Table 1 present the design of the present study.

2.6. Preparation of supernatant

The supernatant of the yogurt samples was acquired through centrifugation (10 g sample at 1613× g for 30 min, at 10°C) according to.[9,15] The supernatant solution was separated and used in the experiment.

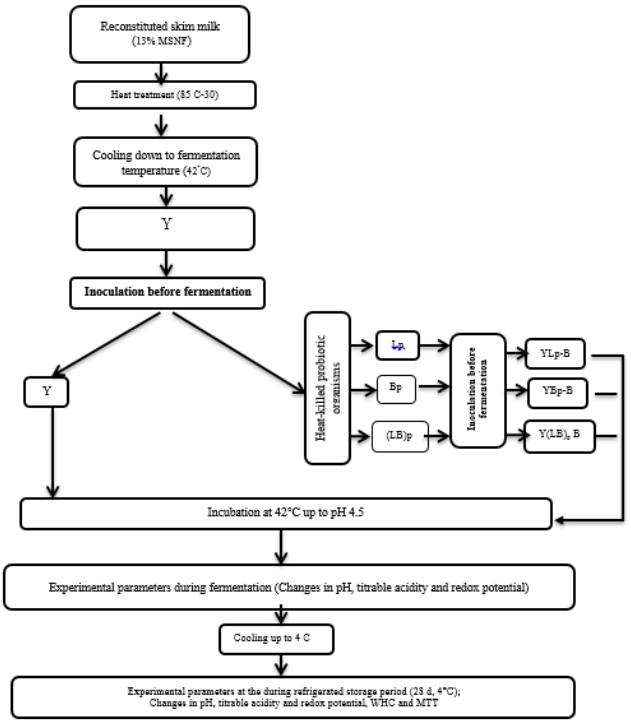

Fig. 1: The study design of present study for single replication

Table 1: Using culture combinations for producing yogurts

Discription of culture component
YLp-B = S. thermophilus + L. delbrueckii ssp. bulgaricus + killed cells of L. acidophilus–before fermentation
YBp-B = S. thermophilus + L. delbrueckii ssp. bulgaricus + killed cells of B. animalis subsp. lactis BB-12–before fermentation
Y(LB)p-B = S. thermophilus + L. delbrueckii ssp. bulgaricus +killed cells of B. animalis subsp. lactis BB-12and L. acidophilus (paraprobiotic) –before fermentation

2.7. Statistical analysis

All experiments were performed in five replicates. Data analyses were done using SPSS. One-way ANOVA (Duncan's test) was used to analyze the significant differences between the means. Statistical significance was reported as '$p< 0.05$'.

3. Results

3.1. Paraprobiotic yogurt supernatant inhibiting the viability of cancer cells

In recent decades, extensive efforts have been made to treat breast cancer. Various treatments are used to treat cancer, including chemotherapy, radiation therapy, hormone therapy, and surgery. Although use of chemotherapy to fight metastatic tumors has the best therapeutic response, the side effects of these drugs along with the resistance of the patient's immune system and resulting damage are among the most important obstacles and limitations in its use this as a treatment method.

Lactobacilli paraprobiotics and their produced postbiotics are composed of a wide range of molecules including surface proteins, cell wall polysaccharides, peptidoglycans, secreted proteins, bacteriocins, and organic acids. Peptidoglycan is particular ingredient of Gram-positive bacteria cell wall. This layer contains of amino acids as well as sugar. The sugar component consists diverse residues of ß (1, 4) linked N-acetylglucosamine and N-acetylmuramic acid. Peptidoglycan acts as a scaffold for fixing the cell envelope, likely protein and teichoic acids. Teichoic acids of L. acidophilus consist of more than 50% of cell wall weight and are various in structure depending on the strain level of growth, medium pH, carbon source, and phosphate. It has different forms such as teichoic acids (TA), teichuronic acids (TUA), lipoteichoic acid (LTA) as well as lipoglycans, while the LTA of L. acidophilus contains glycolipid and polyglycerophosphat. LTA is associated with growth and physiology of L. acidophilus. In this regard, probiotic ruptured cells can have positive effects on the host, such as immunomodulatory, anti-tumor, antimicrobial, and barrier-preservation effects.[16–18] In the present experiment, three supernatants of paraprobiotic yogurts were investigated for their antiproliferative (cytotoxic) effects against three different cancer cell lines. The results of (MTT) dye measurement were obtained by measuring the absorption of light based on the concentration of the supernatant used in comparison with the rate of cell proliferation by drawing a pattern. The percentage of living cells affected by the extract compared to the cells that did not receive the extract was calculated using the following formula:[13]

$$\text{Viability} = \frac{Optical\ absorption\ of\ extract{-}treated\ cells\ in\ each\ well \times 100}{Mean\ light\ absorption\ of\ control\ cells}$$

This laboratory study indicated that the extract had no effect on control cells, while showing a cytotoxic effect on cancer cells according to the dose of the extract and the time of its effect. Table 2 reports the pH values, acidity, and redox potential of the supernatants of the three types of paraprobiotic yogurts. The rate of reduction in pH as well as increase in acidity and redox potential in treatments during the incubation period. The pH values ranged between 0.0060 and 0.0055, with the acidity ranging between 0.20 and 0.25. Furthermore, the redox potential values ranged from 0.33 to 0.37. Table 2 indicates the inhibitory effects of supernatants of paraprobiotic yogurts under diverse biochemical conditions on the growth of cell lines using MTT assay. YBp revealed the lowest acidity increase. The inactivated cells of L. acidophilus had the greatest effect on elevating the rate of acidification and redox potential. Thus, the treatments with the highest rate of acidification had the greatest redox potential due to generation of organic acids

during fermentation The higher buffering capacity observed in YL_p-B compared to other treatments. Greater buffering capacity leads to slower pH drop and stimulates acidification rate by starter bacteria.[19]

Table 2: The pH drop, acidity increase and redox potential increase rates in treatments

Treatments	mpH-DR (pH/day 1)	mA-IR (°D/day 1)	mRP-IR (mV/day 1)
YLp-B	0.0060 ± 0.07^a	0.25 ± 0.06^a	0.37 ± 0.03^a
YBp-B	0.0055 ± 0.04^b	0.20 ± 0.03^c	0.33 ± 0.0^b
Y(LB)p-B	0.0055 ± 0.04^b	0.21 ± 0.05^b	0.33 ± 0.0^b

*Means in the same column shown with different letters are significantly different ($p< 0.05$).

**mpH-DR = mean pH drop rate, mA-IR = mean acidity increase rate, mRP-IR = mean redox potential increase rate

One-way ANOVA analysis revealed YB_p-B could significantly inhibit the cell viability in three cell lines (MDA-Mb 231, SKBR3, and SW 480) and had the greatest effect on SKBR3 ($p= 0.001$). Specifically, at 1% level, a significant difference was observed between the control sample and the concentrations different extracts in all three cell lines at 72 hours. The appearance of cell lines showed that paraprobiotics were effective on them Figure 2 a, b, c.

Furthermore, YL_P-B could inhibit the proliferation of both SKBRT3 and SW480 cell lines respectively ($p= 0.001$ and $p= 0.001$) (Figure 2 a, b). In addition, the supernatant reduced cell viability in MDA-MD231cell line at concentrations of 1/4, 1/3, and 1/2 respectively ($p= 0.05$ and $p= 0.01$) Figure 3 a, b, c. The potency of YL_P-B on breast cell lines was greater than on the colon cell line.

Figure 4 a, b, c displays the Y(LB) p-B effect with the largest inhibitory effect observed at sw480 1/4, 1/3 and 1/2 cell line concentrations ($p = 0.05$ and $p = 0.01$). It could reduce the cell viability on MDA-MD231 and SKBR3 respectively ($p=0.01$ and $p= 0.05$).

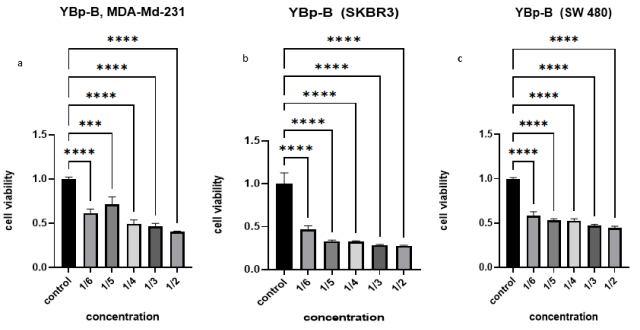

Fig. 2: a, b, c) Survival rate and cell survival of cancer cells treated with different concentrations of supernatant after 72 hours, the results are shown as mean ±standard error

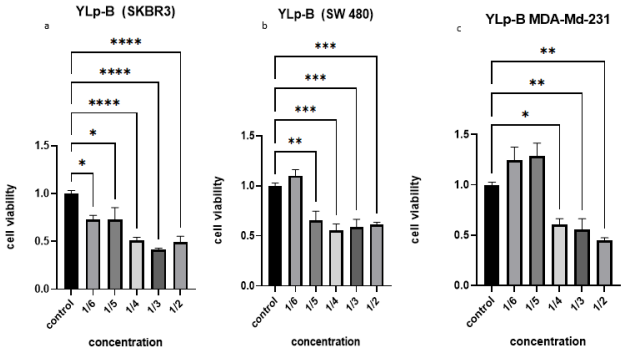

Fig. 3: a, b, c) Survival rate and cell survival of cancer cells treated with different concentrations of supernatant after 72 hours, the results are shown as mean ±standard error

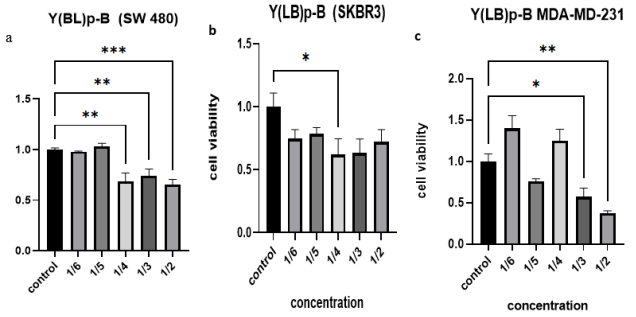

Fig. 4: a, b, c) Survival rate and cell survival of cancer cells treated with different concentrations of supernatant after 72 hours, the results are shown as mean ±standard error

4. Discussion

The human gastrointestinal tract (GIT) contains a rich and complex microbiota, in which compounds and digestive activities play an important role in nutrition, immune system, and certain diseases. There is evidence of the benefits of balance in the gut microbiota for the host health. Under normal circumstances, the composition of the gut microbiota is constant, but it can change due to factors such as changes in diet, medication, and stressful situations.[20] One of the most effective contemporary strategies for maintaining a healthy balance of intestinal microbiota is prescribing probiotics or consuming probiotic foods. Probiotic food is a type of functional food with beneficial effects on human health. Fermentation of bovine colostrum was performed in a two-step method using primer cultures on C. lipolytica and kefir seeds. Extracts of fermented products containing bioactive peptides revealed high angiotensin-converting enzyme (ACE) inhibitory capacity and antioxidant activity. In addition, they presented non-cytotoxic and non-toxic activity as well as potential to stimulate cell proliferation.[21] From among the many functional foods, probiotic yogurt is one

of the most substantial and widely consumed functional dairy products.[22] Yogurt has appropriate matrix for incorporation of probiotics due to its favorable sensory properties and providing essential nutrients. Meanwhile, the production of amino acids and peptides during fermentation creates favorable compounds. Probiotic bacteria in yogurt can generate bioactive compounds with antimicrobial, hypolipidemic, antioxidant, antihypertensive as well as immune-modulatory activity. Saccharomyces boulardii is a probiotic yeast known for its anti-diarrheal effects. Goats' milk yoghurts fortified with Pistacia atlantica resin extract alone or in combination with Saccharomyces boulardii had positive effects on growth of LAB, enhanced the stability of resin phytochemicals, and improved the organoleptic properties.[23] Encapsulation of probiotics of L. acidophilus LA-5 and B. lactis BB-12 with whey protein solution during yoghurt production resulted in a significant reduction in total probiotic count was indicated in yoghurts containing encapsulated probiotics after 21 days of refrigerated storage, where the total number of probiotics increased during in vitro colon fermentation, increasing the biological availability of probiotics in the large intestine.[24] Encapsulation in an alginate–goat milk–inulin matrix could improve the survival of probiotic bifidobacterium animalis subsp. lactis BB-12 in simulated gastrointestinal conditions and goat milk yoghurt.[25] Some research has shown that inactivated probiotics called "paraprobiotics" can yield beneficial outcomes. Paraprobiotic could contribute to release of bacterial compounds including exopolysaccharides, peptidoglycans as well as lipoteichoic acids, resulting in immunomodulating and inhibitory activity against pathogenic microorganisms as well as some diseases (inhibition of the growth of cancer, reduction of lactose intolerance, treatment of atopic dermatitis, modulation of the response to visceral pain, inhibition of dental caries, etc.). Furthermore, nonviable microbial-derived materials can be more advantageous than probiotics due to the feasibility of development of more stable and safer products.[26,27]

Our data revealed that the supernatant YB_p-B could have an inhibitory effect on colorectal and breast cell lines. Studies on inactivated probiotic of Bacillus amyloliquefaciens FPTB16 and Bacillus subtilis FPTB13 at different concentrations (10^7, 10^8 and 10^9 cells ml^{-1}) in a Catla fish revealed that the two inactivated probiotic stimulated the cellular immune response (proliferative response, leukocyte peroxidase content, and respiratory burst activity) of head kidney leukocytes. Also, thermal-treated probiotics and the lowest concentration dose (10^7 ml^{-1}) revealed the supreme stimulation, and in some instances, the stimulatory effect of this inactivated was better than that of the viable cells.[5] Although YL_P-B and Y $(LB)_p$-B could decrease the proliferation rate, their potency was lower than that of YB_p-B for inhibiting cancer cell growth. On the other hand, the present research found that their effect is dose dependent. However, we showed that their effect is dose dependent. The inactivated probiotic of L. casei 01 (by ohmic heating) illustrated hypoglycemic activity both in vitro through the inhibition of α-glucosidase and α-amvlase and in a clinical trial, indicating declined glucose postprandial rates and maintenance of parameters associated with glycemic response, similar to the probiotic whey drink.[28] Several studies have described probiotics as potentially mitigating cancer risk.[29] Cow's milk cedar cheese supernatant showed relatively strong growth inhibition in colorectal cancer cells at concentrations of 400 and 500 μg / ml up to 150 days of cheese maturation and inhibited G0 / G1 phase cell growth. It also induced apoptosis in HCT-116 colon cancer cell line.[30,31] reported that probiotic conjugated linolenic acid has a cytotoxic effect on the cell line MDAMB231 through the downward regulation of NFκB. Another study found that Lactococcus lactis KC24 separated from kimchi has an anticancer effect against gastric carcinoma (AGS), colon carcinoma (HT-29 and LoVo), breast carcinoma (MCF-7), and lung carcinoma (SK-MES-1) cells.[32,33] revealed that Lactobacillus cells and supernatants could have an inhibitory effect on the growth of the HT-29 human colorectal cancer cell line considering elevation of lactate dehydrogenase. Given that cancer is one of the main causes of death worldwide, research on finding new ways of treatment and prevention are necessary for the public health.[34] reported that Prato Matrix dairy cheese had higher nutritional composition (high fat and protein) and higher anti-hyperglycemic activity in vitro (higher alpha-amylase and α-glucosidase inhibitory activity). No changes were observed in other glycemic parameters (PGV, AIg, GIP, GIV, AUC, BG, and peak hyperglycemia). In vitro and in vivo data on products with higher α-glucosidase and α-amylase inhibitory activities (in vitro) would lead to hypoglycemia. In general, the dairy matrix plays an important role in the anti-hyperglycemic index.

Overall, the results of our studies have been in line with other research, and we were able to show that these paraprobiotics could inhibit the growth of colon and breast cancer cells.

5. Conclusion

The results of this study indicated that paraprobiotic yogurt supernatant with anti-cancer effect over time and subtility on cancer cells (MDA-Md, SKBR3, and SW 480) can inhibit the growth of these cells. Gradually and over time at higher doses, it could further inhibit the growth of rat cancer cells.

6. Conflict of Interest

None.

7. Source of Funding

None.

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