In vitro cytotoxic activity of probiotic bacterial cell extracts against Caco-2 and HRT-18 colorectal cancer cells

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Abstract

This research aimed at screening the anti-cancer activity of cell extracts of forty potential probiotic bacterial isolates against 2 colorectal cancer (CRC) cell lines (namely, Caco-2 and HRT-18), and vero cells using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) and Trypan Blue assays (TBE). Results demonstrated that 2 isolates (*Lactobacillus acidophilus* LA102 and *Lactobacillus casei* LC232) showed pronounced cytotoxic activities, with proliferation inhibition of 37% and 68.5% of LA102, and 48% and 45.7% of LC232 against Caco-2 and HRT-18, respectively, at a concentration of 100 µg extract/ml. The IC_{50} values of the cytotoxic activity were 1.6 and 2.5 µg/ml of LA102, and 15.4 and 6.2 µg/ml of LC232 against Caco-2 and HRT-18, respectively. At the same time, results showed that LA102 and LC232 isolates had no cytotoxic effect on the normal vero cells. Even though these observations raise the prospects of using these probiotic isolates for possible cancer prevention and even treatment, yet further investigation is needed to ascertain their potential to prevent *in vivo* human CRC.

Keywords: Probiotic; colorectal cancer; Lactic acid bacteria, Lactobacillus; MTT; cytotoxicity.

Introduction

Cancer is the world's second biggest killer after cardiovascular diseases, with colorectal cancer (CRC) the second cancer cause of death, but also the most preventable cancer type (22). Although significant progress has been made in the field of cancer therapy during last decades, the resistance to chemotherapy becomes a problem in many cases. The diet is likely to play a key role in the pathogenesis of CRC (11). Epidemiological studies have shown that the consumption of red meat and animal fat is associated with an increased risk for CRC development (11), whereas a diet rich in fruits, vegetables, and fibers appears to be preventive against CRC (8). Evidence from a wide range of sources supports the assumption that the link between diet and CRC may be due to an imbalance of the intestinal microflora (10). Therefore, many dietary agents and natural health products have attracted the attention of scientists for their anti-cancer effects, one of these products is the probiotic microbiota, the nonpathogenic microorganisms living in the intestinal tract which benefit the host (8). In human clinical trials, probiotics have shown several proven health effects including modulation of intestinal health and immune system, as well as anti-carcinogenic, anti-diarrheal, hypocholesterolemic effects, and lactose intolerance alleviation (8, 10, 23).

Despite the great number of studies in the literature, the exact probiotic anti-cancer mechanism is still not perfectly explained. However, recently several mechanisms have been proposed including: alteration of the intestinal microflora, inactivation of carcinogenic compounds, anti-proliferative effects via regulation of apoptosis and cell differentiation, improvement of the host's immune response, fermentation of undigested food, and competition with putrefactive and pathogenic microbiota (11, 24). Moreover, the coadministration of probiotics along with prebiotics (indigestible carbohydrates selectively fermented by groups of beneficial bacteria in the intestine, that induce specific changes in the composition and/or activity of the gastrointestinal microflora that confer benefits upon host wellbeing and health) (24), can increase the effectiveness of probiotics anti-cancer activity (15, 20). In addition, the acidification of pH, although not considered as a distinct mechanism of action, is an intrinsic and fundamental feature whereby many probiotics carry out their metabolic activities (18, 25).

Several studies have shown the variation in the probiotic characteristics of isolates from different sources. Human probiotic microbiota differs from an individual to another according to human food habits and types of food consumed. This opens the opportunity of isolating potential probiotic microbiota from individuals of different origins which may have different anti-cancer activities. The aim of this research was to provide information regarding the cytotoxic activity of potential

Table 1: Cytotoxic activity of probiotic isolates *L. acidophilus* LA102 and *L. casei* LC232 cell extract (100 µg/ml) against Caco-2, HRT-18 CRC, and vero cell lines using MTT assay.

Probiotic Isolates	MTT values1 of Cytotoxic activity (Inhibition %)		
	Caco-2	HRT-18	Vero cells
***L. acidophilus* LA102**	0.160±0.007[2] (37±1.5%[3b4])	0.312±0.005[2] (68.5±2.8%[a])	0.329±0.005[2] (-3.62±0.11%[a])
***L. casei* LC232**	0.208±0.011[2] (48±1.3%[a])	0.208±0.008[2] (45.7±2.2%[b])	0.332±0.009[2] (-4.73±0.06%[a])
	0.433±0.005[5]	0.456±0.0012[5]	0.317±0.011[5]

1 All values are mean ± SD of 3 experiment.

2 Absorption values of MTT assay of CRC cell lines treated with probiotics cell extracts.

3 Inhibition % was calculated as the following= {1– [(absorbance of sample)/(absorbance of control)]} × 100.

4 Means within the same column followed by different superscripts are significantly different by ($P < 0.05$).

5 Absorption Values of MTT assay of CRC cell lines untreated with probiotics cell extracts.

probiotics isolated from the guts of Jordanians against different human CRC cell lines (Caco-2 and HRT-18), alongside determining IC_{50} values.

Materials and methods

Preparation of Probiotics Cell Extract:

Forty potential probiotic bacterial isolates were grown in MRS broth (Oxoid, UK) supplemented with 0.5% cysteine-HCl, and then incubated at 37°C for 18 hours under anaerobic conditions in an aerobic jar (Oxoid). The MRS broth was inoculated with 10^{5-6} cfu/ ml probiotics culture (5). After incubation, the cultures were sonicated 4 times for 20 second with freezing cycles in between for 4 minutes, then harvested by centrifugation (5000 g/15 min/2°C). The pH of the cell-free supernatant was adjusted to 7 with 0.1M NaOH. The cell-free supernatant was filter-sterilized with a micro-filter (0.22 µm; Millipore Ltd., Hertfordshire, England) and concentrated using a rotational vacuum concentrator, then stored at 2°C until used in the assay (25).

Colorectal Cancer and vero Cell Lines Culture:

Two CRC cell lines (Caco-2 and HRT-18 cells) and African green monkey kidney (Vero) cells (American Type Collection, USA), were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) and Roswell park memorial institute (RPMI) 1640 medium, respectively. The media were supplemented with 25 mM glucose, 10% inactivated fetal bovine serum (Flow, McLean, VA, USA), and 1% penicillin/streptomycin, and maintained at 37°C in a humidified incubator with 5% CO2. The cells with 30-40 passages were used for further investigations (3).

Cytotoxic activity (MTT Assay) and IC_{50} values of potential probiotics cell extracts:

The screening of cytotoxic (anti-cancer) activity of 40 potential probiotics cell extracts was carried out using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). The assay measured the formation of blue formazan product as a result of the reduction of MTT by mitochondrial dehydrogenase, which indicates the normal function of mitochondria and cell viability (2, 14). Exponentially growing cells have been washed and seeded at $2x10^4$ cells/well (100 µl/well) in 96 well microplates (Greiner, Germany). Concentrated crude probiotic cell extract was added (100 µg/well) to the wells of each cell line beside Vero cell lines. Cells were incubated at 5% CO2 concentration for 72h at 37°C. Then 20 µl of PBS containing 5mg/ml MTT was added in each well. After 4 h contact with the MTT solution at 37°C, formazan blue crystals formed and then they were dissolved in 100 µl DMSO. Reduced MTT was measured at 570nm using a microplate reader (Biotek, USA). Untreated cells were used as a negative control. The cytotoxicity of the tested extracts was determined by comparing the absorption of the treated cells against the absorption of the control (untreated cells). This was expressed as: {1– [(absorbance of sample)/(absorbance of control)]} × 100, as the median of 3 independent experiments. For the determination of IC_{50} values, probiotic cell extracts were dried, weighed and serially diluted in RPMI 1640 media, to achieve the following concentrations (200, 150, 100, 50, 25, 12.5, 6.25 µg/ml), and added (100 µl/well) to be tested against Caco-2 and HRT-18 cell lines. Above mentioned MTT assay was used to determine IC_{50} values, that were calculated as the concentrations that show 50% inhibition of any tested cell line.

Viability of CRC Cell Lines (TBE Assay):

Trypan blue exclusion (TBE) assay was used to determine cell viability every 24 h for three days (2, 14). This test is based on the fact that viable cells do not take up Trypan blue dye, whereas dead cells are permeable to the dye. Caco-2 and HRT-18 cell suspension ($1x10^5$ cells/ ml) were seeded in 24 well micro titer-plates, treated with the concentration of the IC_{50} values and incubated at 37°C for 72 h. Then, 20 µl suspension were collected from each well and stained with equal volume of Trypan blue, then counted using Hematocytometer, every 24 h (2). The number of stained cells and the total number of cells were counted and the percentage of viable cells was measured using the following formula:

Viability % = No. of cells with treatment /No. of cells without treatment × 100

Results and discussion

Current study aimed at screening the cytotoxic activity and IC_{50} values of probiotics cell extracts against Caco-2 and HRT-18 cell lines using MTT assay, and further to determine best dose-time exposure combination using TBE assay. The current study showed various degrees of cytotoxic activities of different probiotics extracts against both CRC cell lines (data not shown). Among the tested probiotic isolates, *L. acidophilus* LA102 and *L. casei* LC232 showed the highest cytotoxicity against Caco-2 and HRT-18 cell lines (Table 1). The cytotoxicity values were 37% and 68.5%; 48% and 45.7% of LA102 and LC232 against Caco-2 and HRT-18 cell lines, respectively. This cytotoxic effect was approved by the reduction in MTT absorption readings (Table 1). The MTT absorption readings of Caco-2 reduced from 0.433 to 0.160 and 0.208 for untreated, LA102 and LC232 treated cell lines, respectively; whereas the absorption readings of HRT-18 reduced from 0.456 to 0.312 and 0.208 for LA102 and LC232 treated cell lines, respectively.

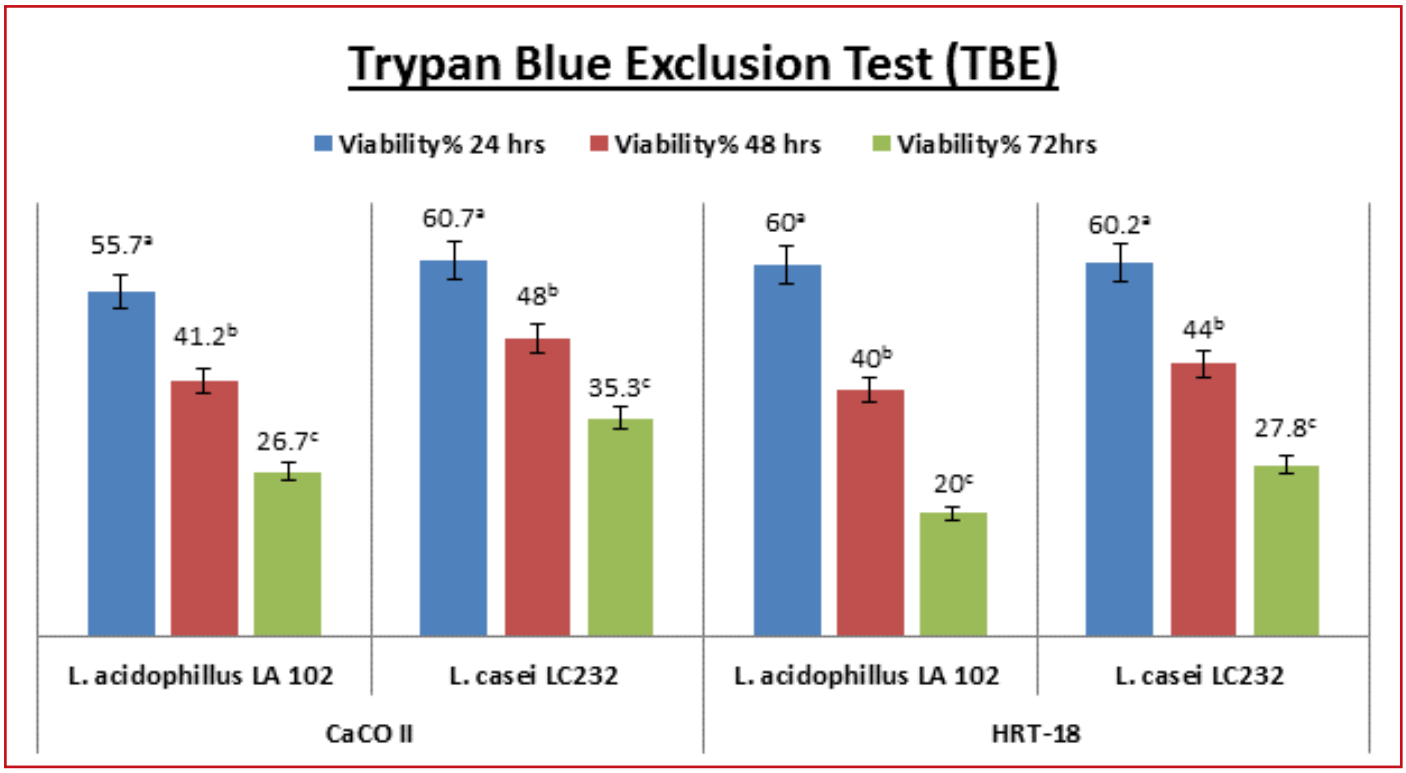

Figure 1: Time effect of the IC_{50} concentrations of probiotic cell extracts on the growth of cells after 24, 48 and 72h. Cell viability quantified by using Trypan blue assay. Results are average of triplicate independent experiments. Error bars represent mean ± SD. Means within the same treatment followed by different superscripts are significantly different by ($P < 0.05$).

Whereas the growth of Vero normal cell line was not significantly inhibited by 100 µg/ml of each cell extract, as the MTT absorption readings of the treated cells was marginally higher than untreated cells after 72 h of incubation (Table 1).

The IC_{50} values showed that LA102 isolate had higher cytotoxic effects against Caco-2 and HRT-18 than LC232 isolate (Table 2). The IC_{50} values were 1.6 and 2.5 µg/ml and 15.4 and 6.2 µg/ml for LA102 and LC232 against Caco-2 and HRT-18 cells, respectively. Moreover, results of TBE assay determined the optimal exposure time of the cytotoxic effect of cell extract on Caco-2 and HRT-18. Results shown in Fig 1 demonstrated that the highest cytotoxicity showed by LA102 and LC232 on Caco-2 and HRT-18 cell lines were after 24 and 72 h, respectively. Results showed that the viability of Caco-2 cells was reduced by 44.3%, 58.8%, and 73.3% by LA102; and by 39.3%, 52.0%, and 64.7% by LC232 after 24, 48, and 72 h, respectively. Whereas, the viability of HRT-18 cells was reduced by 40.0%, 60.0%, and 80.0% by LA102; and by 39.8%, 56.0%, and 72.2% by LC232 after 24, 48, and 72 h, respectively.

Probiotic bacteria have been associated with a variety of health benefits including enhanced coronary heart health, immune performance, suppression of tumor cells and protection against colon cancer (4, 14). Anti-cancer activity of probiotic bacteria is one of the most recently studied fields of probiotics health-promoted effects (1, 29). Regardless of the availability of a large quantity of data in the literature, the exact mechanisms by which probiotics may prevent CRC is still not well established. However, it is conceivable that they include: inhibition of the tumor cell proliferation and induce apoptosis; inhibition the production and activity of many carcinogenic enzymes, such as β-glucuronidase and nitroreductase, anti-mutagenicity; modulation of the immune response to tumor tissue; and binding of certain mycotoxins and cyanobacterial toxins (1, 22).

Our results demonstrated that two potential probiotic isolates (LA102 and LC232) exerted maximum cytotoxicity against CRC cell lines. Results of MTT and TBE assays were marginally the same, with a small difference in the results, which could be as a result to the difference in the principle of each assay. Our findings are in agreement with Roy et al. (26), who reported the similarity of results obtained by MTT and TBE assays. The results presented in this study provide further evidence for the anti-cancer activities of the probiotic bacteria, and these results reflect the high potentiality of the cytotoxic activity of both isolates, and consequently the potential for CRC prevention. LA102 demonstrated a higher significant cytotoxic activity than LC232 (Table 1 and Fig. 1), as confirmed by the lower IC_{50} values of LA102 compared to LC232 (Table 2), with keeping in mind that both isolates had relatively low IC_{50} values when compared to IC_{50} values found in the literature. For example, Phonnok (21) showed that the IC_{50} values of cytotoxicity of 394 bacterial isolates ranged from 21.1 to 438µg/ml against several cancer cell lines. Moreover, Boik (6) stated that a crude extract is generally considered to have in vitro cytotoxic activity if the IC_{50} value of a 48–72h pretreated carcinoma cells is less than 20µg/ml, while it is less than 4µg/ml for pure compounds.

Several probiotic strains have been shown to exert different mechanisms that are related to the cytotoxic activity. For example, Lee et al. (16) concluded that *L. acidophilus*, *L. casei* and B. longum have possessed immunomodulatory and antitumor effects by suppressing the proliferation of tumor cells. In addition, several other strains of LAB have been shown to exert strong cytotoxic effects, such as *L. casei* Shirota, which exerted strong anti-metastatic effects on transplantable tumor cells and to suppress chemically induced carcinogenesis (28). However, the available literature regarding probiotics anticancer activity revealed that the effect of live cells is the most studied field, and to a lesser extent the effect of probiotics cell extract, with keeping in mind that a considerable amount of ingested probiotic cells is killed

Table 2: The IC_{50} of probiotic isolates against CRC cell line Caco-2 and HRT-18 cells using MTT assay.

Probiotic Isolates	IC_{50} (µg/ml)[1]	
	Caco-2	HRT-18
***L. acidophilus* LA102**	1.60±0.05[2,b3]	2.50±0.04[b]
***L. casei* LC232**	15.4±0.29[a]	6.20±0.25[a]

1 IC_{50}: The median lethal concentration

2 All values are mean ± SD of 3 experiment

3 Means within the same column followed by different superscripts are significantly different by ($P < 0.05$).

and lysed within the digestive tract. Accordingly, in our study we aimed at studying the cytotoxic effect of whole lysed cell fractions in order to mimic the status of probiotic bacteria lysis and action in the digestive tract. The cell fractions resulted from lysis is believed to include cell wall peptidoglycan and cytoplasm fractions. The cytotoxicity of different probiotics cell fractions, such as whole cells, heat-killed cells, the cell wall, peptidoglycan, and cytoplasmic fraction, all have been reported against different human cancer cell lines (13). For example, peptidoglycans isolated from *L. casei* were found to have anticancer activities against different human cancer cell lines including Caco-2 (9). In addition, it was reported that polysaccharide and glycoproteins fractions originating from *Lactobacillus* cultures had showed the same cytotoxic effects (17, 19). In a study by Sevda et al. (27), the antiproliferative effects of the cell-free filtrate and the cell-free lyophilized filtrate of LAB cultures was reported against Caco-2 cell lines. Also, Choi et al. (7) reported that the antiproliferation effect of heat killed cultures of *L. acidophilus* 606 and *L. casei* ATCC 393 against different human cancer cell lines, including CRC cell lines (HT-29) was due to the soluble polysaccharides fraction of the cell wall. Finally, although induction of apoptosis in CRC cell lines by probiotics has been well reported (2, 4), in current study the killing mechanism has not been determined and further investigation is required to define the mechanism of inhibition as apoptosis or necrosis.

Conclusion

In conclusion, among the forty probiotics cell extracts investigated in the current study, only *L. acidophilus* LA102 and *L. casei* LC232 exhibited a strong cytotoxic activity against Caco-2 and HRT-18 CRC cell lines. These findings seem to be a promising approach for use of some probiotic strains isolated from human origin as a support therapy or prevention of cancer. Additional investigation of probiotics cytotoxic mechanisms of action, whether apoptosis or necrosis, and *in vivo* studies are necessary to ascertain if results obtained from in vitro experiments can be translated to the clinical practice.

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