Original Article

The Inhibitory Effects of *Lactobacillus Reuteri*'s Cell Wall on Cell Proliferation in the HCT-116 Colorectal Cancer Cell Line

Zahra Rafiei Atani[1], Ebrahim Faghihloo[1], Zohreh Ghalavand[1], Gita Eslami[1*]

[1] Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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Abstract

Background: Colorectal cancer (CRC) is the third and a second common cancer in men and women respectively in the world and about 1.4 million new cases diagnosed in 2012. The normal gut microflora consists of bacterial species. One group of them is probiotics, which confer a health benefit to the host. Lactobacillus *reuteri* (*L.reuteri*) is known as a probiotic, which lead to the prevention of colorectal cancer. The aim of this study was to assess the inhibitory effects of Lactobacillus reuteri's cell wall on cell proliferation in the colorectal cancer HCT-116 cell line.

Materials and Methods: The cells of HCT-116 cell line were grown at 37°C, 5% CO2. *L.reuteri* was obtained from the Iranian Biological Resource Center and cultured in the MRS Broth at 37°C for 48h anaerobically. The cell wall was prepared by the freezing-thawing procedure. So the inhibitory effect of *L.reuteri* on the growth and proliferation of HCT-116 cells was assessed by MTT assay.

Results: The cell wall from *L.reuteri* inhibited cell proliferation on colorectal cancer HCT-116 cell line. It showed dose- and –time dependent inhibition.

Conclusion: These results demonstrated that cell wall of *L.reuteri* inhibits cell proliferation of HCT-116 cell line.

Keywords: Colorectal cancer, *Lactobacillus reuteri*, HCT-116 cell line

***Corresponding Author:** Gita Eslami, PhD; Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Tel: (+98) 21 2387 2556; Fax. (+98) 21 22439972; Email: eslami202@sbmu.ac.ir

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Introduction

Colorectal cancer (CRC) is the third and a second common cancer in men and women respectively in the world and about 1.4 million new cases diagnosed in 2012[1-4]. In the United States in 2017, it is anticipated to be 135,430 individuals newly diagnosed with CRC and 50,260 deaths from the disease[5]. CRC is the third and fourth common cancer in men and women respectively in the Iran[6].

The risk factors of this disease are varied. Based on epidemiological studies, the consumption of red meat and animal fat is associated with an increased risk for CRC development, whereas a diet rich in fruits and vegetables appears to be protective against this illness[7,8]. Some studies support that the large and complex bacterial population hosted by the large intestine and known as "gut microbiota" plays a critical role in the CRC[8,9].

The normal gut microflora consists of bacterial species with morphological, physiological and genetic features that coexist with other colonizing microorganisms and competitively inhibit the growth of pathogenic bacteria. Nevertheless, some environmental factors

such as diet and drugs can alter the composition of the resident microbiota, with consequences for the health of the host[10]. One group of them is probiotics, which confer a health benefit to the host when administered in adequate amounts[8]. Probiotics seem to be one of the most interesting candidates for the treatment of colorectal cancer[11]. *In vitro* and *in vivo* studies as well as epidemiological studies have shown the positive role of probiotics against cancer[12,13].

Some Lactic acid bacteria (LAB) strains like *Lactobacillus reuteri* (*L.reuteri*) is known as probiotics, stimulate the immune system, leading to the prevention of colorectal cancer[14]. LAB are Gram-positive, non-sporulating, nonmotile, heterotrophic anaerobic or facultative aerobic cocci or rods, which produce lactic acid as one of the main fermentation products of the metabolism of carbohydrates[15]. In this present study, we aimed to evaluate the effects of *L.reuteri*'s cell wall to inhibit the proliferation and growth of colorectal cancer HCT-116 cell line.

Methods

Cell culture: Colorectal cancer cell line (HCT-116) was purchased from the Pasteur Institute of Iran (National Cell Bank department), cultured in RPMI1640 medium containing 100 µg/ml Streptomycin and 100 units/ml Penicillin with 10% fetal bovine serum (FBS) and cells were grown at 37°C, with 5% CO_2.

Bacterial culture and preparation of cell wall: *L.reuteri* was obtained from the Iranian Biological Research Center (IBRC-M10755) and cultured in the de Man, Rogosa and Sharp medium (MRS Broth-Conda S.A. Spanish) at 37°C for 48h anaerobically by Whitley Jar Gassing System (Don whitely scientific- Britain). For the *in vitro* preparation of the bacterial cell wall, cultured cells was centrifuged at 3500 rpm for 15 minutes at 4°C. Sediments were treated with 1ml PBS and centrifuged at 3500 rpm for 15 minutes at 4°C for two times then stored at -70°C overnight. 1ml lysis buffer (Glycerin 10%, Triton X-100 1%, Lysozyme 0.2mmol and PBS), were added to the sediments, in some cases freez-thaw was done, then sonicated (dr.hielscher, Germany) at 60% amplitude for 1 min afterward centrifuged 13500 rpm for 30 min at 4°C. The cytoplasmic (supernatant) and cell wall (sediment) were prepared and stored at -70°C overnight, then samples were dried (Freeze dryer, Christ, Germany) at -59°C for 5h. So various concentrations of cell wall (125, 250, 500, 1000, 2000 and 4000 µg/ml) was prepared[16].

Microculture Tetrazolium Test (MTT assay): The inhibitory effect of *L.reuteri* on the growth and proliferation HCT-116 cells was assessed by MTT assay. Briefly, 25000 cells were plated in 96-well plates until they reached 80% confluence. After 24, 48 and 72 h incubation, cells were treated with different concentrations of the cell wall. Then MTT solution was added to each well and the plates were incubated for four hours at 37°C. After solubilization of precipitated formazan by adding 200µl DMSO and incubating at 37°C for 15 minutes. The optical density was measured at 570 nm. The inhibition rate (IR) of *L.reuteri* was evaluated using the following equation[17]:

OD_{exp} / average OD_{con} × 100.

Results

L.reuteri's cell wall treatment of cells leaded to inhibit cell proliferation. Our study showed that the inhibitory effect of *L.reuteri*'s cell wall on HCT-116 cell line. It was detected by MTT assay for 24, 48 and 72 hours. Death rate of cells was assessed as relative to untreated control cells. Death of cells increased with high dose and time. The cell wall dose of 500 µg/ml survived cells in 24 hours (IC_{50} = 51.15%) and in this dose of cell wall the cell viability percentages were 37.2625% and 31.1525% in 48 and 72 hours, respectively. While the cell viability percentages with cell wall dose in 250 µg/ml were 69.8325% and 54.65% in 24 and 48 hours, respectively, and in this dose of cell wall survived cells in 72 hours (IC_{50} =50.14%), Figure 1 (A, B, C and D).

Discussion

Our study showed that *L. reuteri*'s cell wall prevented cell proliferation on HCT-116 cell line. Inhibitory effects of the bacterial cell wall were increased by expanding in dose and time.

A number of studies indicated that inhibitory effects of

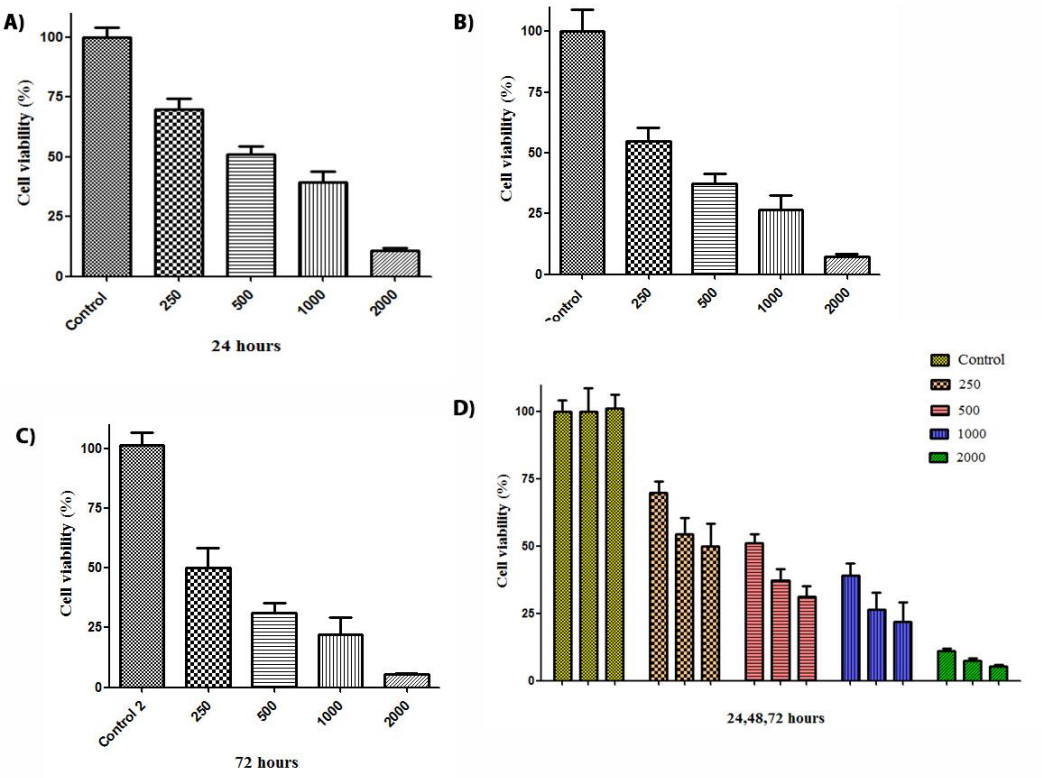

Figure 1. The inhibitory effect of *L.reuteri*'s cell wall on cell proliferation in the HCT-116 colorectal cancer cell line.
A) Cell viability percentages treated with different doses of cell wall on cell proliferation in 24 hours, 250 µg/ml (69.8325%), 500 µg/ml (51.15%), 1000 µg/ml (39.245%) and 2000 µg/ml (10.93%).
B) Cell viability percentages treated with different doses of cell wall on cell proliferation in 48 hours, 250 µg/ml (54.65%), 500 µg/ml (37.2625%), 1000 µg/ml (26.4325) and 2000 µg/ml (7.325%).
C) Cell viability percentages treated with different doses of cell wall on cell proliferation in 72 hours, 250 µg/ml (50.14%), 500 µg/ml (31.1525%), 1000 µg/ml (21.9725%) and 2000 µg/ml (5.36%).
D) Cell viability percentages treated with different doses of cell wall on cell proliferation in 24, 48 and 72 hours.

LAB were vary in different cell lines with different supernatant doses. Yuna *et al*, showed that *B. adolescentis* SPM0212 cell-free supernatant inhibited the growth of SW480, HT-29, and Caco-2 cells. The cell viability percentages with different doses of supernatant in 25, 50, 100 and 200 µg/ml were less than 50% in 72 hours[18]. While our study showed that the cell viability percentages with the cell wall doses in 250 µg/ml was more than 50% and in 500, 1000 and 2000 µg/ml were less than 50% in 72 hours on HCT-116 cell line. Yuna concluded that *B. adolescentis* SPM0212 inhibited TNF-α production, changes in cellular morphology and harmful fecal enzymes[18]. In addition, Angeliki Tiptiri-Kourpeti showed that anti-proliferation effects of increasing concentrations of *L. casei* cell-free-supernatant (CFS) at different time points on murine CT26 and human HT-29 colon cancer cells was vary. The cell viability percentages with CFS doses in 400 and 800 µg/ml were less than 50% in CT26 and of 100, 200, 400 and 800 µg/ml were more than 50% in HT-29 cells at 72 hours [19]. While our study showed that the cell viability percentages with *L. reuteri*'s cell wall doses in 250 µg/ml was more than 50% and in 500, 1000 and 2000 µg/ml were less than 50% in 72 hours on HCT-116 cell line. These findings provide evidence for beneficial tumor-inhibitory, anti-proliferation and proapoptotic effects driven by this probiotic LAB strain[19]. A study have also indicated that the administration of other lactobacillus strains such as, the supernatants of *lactobacillus acidophilus* and *lactobacillus casei* reduced cell proliferation and increased cell apoptosis on $CaCo_2$[20].

As well, *Lactobacillus delbrueckii* fermentation (LBF) solution inhibited the growth of colorectal cancer SW620 cells. This study concluded that the LBF

solution induced apoptosis through the intrinsic caspase 3-depedent pathway, with a corresponding decreased expression of Bcl-2. The activity of matrix metalloproteinase 9, which is associated with the invasion of colorectal cancer cells is decreased in the LBF-treated cells[21].

Other studies have showed that *Bifidobacterium bifidum* supernatant inhibited the proliferation of $Caco_2$ cells [20, 22]. In this study the cell viability percentages with *Bifidobacterium bifidum* supernatant doses in 100, 200 and 300 µl/ml were less than 50% in 24, 48 and 72 hours, while our study showed cytoplasmic extraction doses of 250 and 500 µg/ml survived cells less than 50% in 24, 48 and 72 hours. A similar study reported anti-proliferation effects of *L. rhamnosus GG* (*L. GG*) on gastric and colorectal cancer cells. This study showed that increasing concentrations of *L. GG* homogenate and cytoplasmic extraction reduced the percentages of cell viability to nearly 55% and 65% in DLD-1 (colon) and HGC-27 (gastric) cancer cell lines, respectively. Both HGC-27 and DLD-1 cells were resistant to the bacterial cell wall fractions, whereas induced anti-proliferation effect by increasing cytoplasm fraction concentrations [23]. These data suggest that cytoplasmic extraction may be responsible for *L. GG* action on proliferation in these two cell lines from gastric and colonic neoplasms[23]. In addition, another study showed the cytotoxicity effect of cell wall extracted from *Lactobacillus casei* and *paracasei* on K562 cell line. In mentioned study, the cell viability percentages with *Lactobacillus casei*'s cell wall doses in 2000 and 4000 µg/ml were less than 50% and in 500 and 1000 µg/ml were more than 50% in 24, 48 and 72h. The cell viability percentages with *Lactobacillus paracasei*'s cell wall doses in 2000 and 4000 µg/ml were less than 50% and in 500 µg/ml were more than 50% in 24, 48 and 72h. The 1000 µg/ml was less than 50% in 24h and more than 50% in 48 and 72h. The 500 µg/ml was more than 50% in 24h and less than 50% in 48 and 72h[24]. While our study showed that the cell viability percentages with *Lactobacillus reuteri*'s cell wall doses in 250 µg/ml is more than 50% and in 1000 and 2000 µg/ml are less than 50% in 24, 48 and 72h.

Conclusion

Our study demonstrated that cell wall of *L. reuteri* inhibited cell proliferation of HCT-116 cell line. The inhibitory effect increasing with increased dose and time.

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