RESEARCH ARTICLE

Lactobacillus acidophilus and *Lactobacillus crispatus* Culture Supernatants Downregulate Expression of Cancer-testis Genes in the MDA-MB-231 Cell Line

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Abstract

Lactobacilli are probiotics shown to have antitumor activities. In addition, they can regulate gene expression through epigenetic mechanisms. In this study, we aimed to assess anti tumor activities of *Lactobacillus acidophilus* and *Lactobacillus crispatus* on the MDA-MB-231 breast cancer cell line. The effects of culture supernatants were determined by MTT [3-(4,5-dimethylthiazol-2-y-2,5-diphenyltetrazolium bromide] assay. Changes in expression of 5 cancer-testis antigens (CTAs), namely AKAP4, ODF4, PIWIL2, RHOXF2 and TSGA10 ,were analyzed by quantitative real time RT-PCR. The culture supernatants of the 2 lactobacilli inhibited MDA-MB-231 cell proliferation. In addition, transcriptional activity of all mentioned CTAs except AKAP4 was significantly decreased after 24 hour treatment with culture supernatants. This study shows that *Lactobacillus acidophilus* and *Lactobacillus crispatus* have antiproliferative activity against MDA-MB-231 cells. In addition, these lactobacilli could decrease transcriptional activity of 4 CTAs. Previous studies have shown that expression of CTAs is epigenetically regulated, so it is possible that lactobacilli cause this expression downregulation through epigenetic mechanisms. As expression of CTAs in cancers is usually associated with higher grades and poor prognosis, downregulation of their expression by lactobacilli may have clinical implications.

Keywords: MDA-MB-231 - *Lactobacillus acidophilus* - *Lactobacillus crispatus* - cancer-testis antigens

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Introduction

Lactobacilli are a group of beneficial organisms called probiotics which have a health benefit for the host when administered in sufficient amounts (Isolauri, 2001). They are the normal flora of healthy human vagina and have an important function in protecting the host from urogenital infections (McLEAN and Rosenstein, 2000). The mechanism of action of lactobacilli seems to be strain specific (Otles et al., 2003). *Lactobacillus crispatus* and *Lactobacillus acidophilus* are among the most commonly occurring species in vagina of healthy women (Vásquez et al., 2002; Motevaseli et al., 2013b). Probiotic bacteria have shown to have antineoplastic functions (Meurman et al., 1995), but their exact mechanism of cell growth inhibition is not fully elucidated. *Lactobacillus crispatus* has shown cytotoxic effects on cervical tumor cells, but not on normal cells (Motevaseli et al., 2013a). Lactobacilli have demonstrated the ability to control pathogens overgrowth and modulate systemic inflammation, cell proliferation, and apoptosis (Motevaseli et al., 2013a). In addition, they have been shown to regulate gene expression by simultaneous decrease in histone acetylation and increase in DNA methylation (Ghadimi et al., 2012). Cancer-testis antigens (CTAs) are a group of tumor associated antigens with restricted expression in normal tissues except testis and expression in different tumors (Seifi-Alan et al., 2013). Their expression has been shown to be epigenetically regulated (Ghafouri-Fard and Modarressi, 2009). As lactobacilli may exert their antitumor functions through epigenetic mechanisms, we evaluated expression of 5 CTAs named AKAP4, ODF4, PIWIL2, RHOXF2 and TSGA10 in breast cancer cell line MDA-MB-231 after treatment with *L. acidophilus* and *L. crispatus* culture supernatants.

AKAP4 has been shown by microarray gene expression analysis to be expressed only in testis among normal tissues and in various cancer cells (Hofmann et al., 2008). AKAP4 has been suggested as a serum based biomarker for early detection of breast cancer and a potential target for immunotherapeutic use (Saini et al., 2013b). ODF4 is a testis specific gene which has been shown to be expressed

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in chronic myeloid leukemia patients (Ghafouri-Fard et al., 2012). PIWIL2 has been shown to be expressed in precancerous stem cells, tumor cell lines and a variety of human cancers including breast cancer. Its expression has been shown in various stages of breast cancers and it has been suggested as a novel biomarker (Liu et al., 2010). RHOXF2 is a known CTA (Ghafouri-Fard et al., 2012) and stem cell marker with expression in a range of cancer cell lines and tumor samples (Shibata-Minoshima et al., 2012). TSGA10 is a CTA with expression in a wide variety of tumors including breast cancer. It has been suggested to have role in proliferation and survival of breast cancer cells (Dianatpour et al., 2012).

Materials and Methods

Cell culture

Human breast cancer cell line (MDA-MB-231) was obtained from the Pasteur Institute, National Cell Bank of Iran. Cells were cultured in RPMI 1640 medium containing 10% heat inactivated fetal calf serum (Invitrogen), 1.5% HEPES (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells were maintained as monolayer cultures at 37ºC in a humidified 5%CO_2 atmosphere, and were plated 24 h before treatment to allow adherence.

Preparation of supernatants from Lactobacillus cultures

L. crispatus strain SJ-3C-US (LbC) and L. acidophilus strain La5 (LbA) were grown in de Man Rogosa Sharpe (MRS) broth (Merck; pH 6.5) at 37ºC for 24h under microaerophilic conditions. MRS has a rich nutrient base, polysorbate, acetate, magnesium and manganese, to enhance the growth and proliferation of lactobacilli. Overnight bacterial cultures contained 2×10^9 c.f.u./ml, and these cultures were centrifuged at 1100 g for 15 min at 4ºC. The resulting lactobacilli supernatants (LS) were filtered through a 0.2 mm membrane filter to remove the remaining bacteria and debris. The pH of the LS was 4±0.1, and decreased from 6.5 (MRS broth pH). To test whether lactate produced by the two lactobacilli and pH change would affect tests, the lactate concentration in LS was checked using a Lactate Randox kit (Randox Laboratories); noncultured MRS broth adjusted to pH 4 with lactate was used in co-culture tests. The following were tested: LbA supernatant, pH 4 (LAS); LbC supernatant, pH 4 (LCS); MRS, pH 6.5; MRS pH 4, adjusted with lactate (MRL).

MTT assay

Cell growth inhibition was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit (Sigma). A total of 2×10^4 cells were seeded in each well containing 100 ml standard medium. After overnight growth, cells were treated for 24h with 1, 2, 5, 10, 15, 20, 40, 60, 80 and 100% (v/v) lactobacilli culture supernatants. Plates were incubated at 37ºC under 5% (v/v) CO_2. Cell viability was determined as follows: ***viability (percentage of control)=[(absorbance sample-absorbance blank)/[absorbance control-absorbance blank)]×100***

RNA isolation, cDNA synthesis and quantitative RT-PCR (qRTPCR)

The FastPure RNA kit (Takara Bio) was used to isolate total RNA from cultured cells. RNA concentration was assessed spectrophotometrically using a Nanodrop 2000c spectrophotometer (Thermo Scientific). Changes in mRNA expression of desired genes were analyzed by quantitative PCR (qPCR) after reverse transcription of 1 mg RNA from each sample with the PrimeScript RT reagent kit (Takara Bio). qRT-PCR was performed for mRNA quantification of genes on a rotor gene 3000 corbette detection system using SYBR Premix Ex Taq (Takara Bio). Primer sequences are listed in Table 1. Thermal cycling conditions were an initial denaturation at 95ºC for 1 min, and 40 cycles at 95ºC for 15s and 65ºC for 1 min. The PCR was performed in a final volume of 20 ml containing 10 ml SYBR Green master mix, 2 ml cDNA, 0.5 ml each forward and reverse primer (10 pmol) and 7 ml nuclease-free water. Experiments were performed in duplicate for each data point. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was amplified as a normalizer, and fold changes in each target mRNA expression relative to GAPDH were calculated. Melting curve analysis was used to validate whether primers yielded a single PCR product.

Statistical analysis

The total expression ratio of the cancer-testis genes was compared between treated and control cells using a randomization test implemented in the relative expression software tool, which is an Excel-based application for group-wise comparison and statistical analysis of relative expression findings of qRT-PCR.

IC_{50} (concentration giving half-maximal inhibition) of cells treated with lactobacilli culture supernatants were compared with pH- and lactate adjusted and pretreated controls using the Mann–Whitney test with SPSS software. All data were expressed as a mean±SE of three separate experiments. $p<0.05$ was considered as statistically significant.

Results

*Supernatant of **L. acidophilus** (LAS) and supernatant of **L. crispatus** (LCS) inhibit MDA-MB-231 cell proliferation*

Cell growth inhibition was measured by MTT assay. The IC_{50} value of LAS and LCS against MDA-MB-231 cells was 15% (v/v). The cytotoxic effects of LAS and LCS against MDA-MB-231 cells are higher than those of MRS and MRL (MRS with pH adjusted to that of LAS and LCS) (Figure 1). These results showed that the main cause of MDA-MB-231 cell death was not the acidity. It

Table 1. Primer Sequences

Gene	Product length	Forward primer	Revers primer
AKAP4	221	GGCAGTCAAGGCTGTAGGAG	GCTGTCCTTCTGGGTTGTAGAG
GAPDH	199	CGGCAGCATCAAATGTTTCAG	CGGCAGCATCAAATGTTTCAG
ODF4	250	AATTGTGGAATTGATGCGTGATG	CTACAACGATCCCCTCTGAAAAA
PIWIL2	168	CTTCGTGGCAAGCATCAATC	TCACGGTACACCACAATCTTC
RHOXF2	191	GCTACTGCCCCACCATGACC	ATGGACTCGAAGCGCACATC
TSGA10	252	CAACGGCACATGCTATTCTCC	CCACAGTGCTTATGGTTTCCTTC

could be the result of a substance other than lactate in the supernatant of the lactobacilli that causes breast tumor cell death.

Expression of CTAs in MDA-MB-231 cell line

All 5 mentioned CTAs have been expressed in MDA-MB-231 cell line. Normal testis sample was used as positive control (Figure 2).

LAS and LCS downregulate mRNA of CTAs

mRNA levels of 5 mentioned CTAs in MDA-MB-231 were measured by qPCR before and after treatment with LAS and LCS. After 24 h treatment of MDA-MB-231 cells with 10% (v/v) LAS, LCS and MRS, LAS and LCS downregulated mRNA levels of all genes. This downregulation was statistically significant, when compared to MRS and MRL, for all genes except for AKAP4 (Figure 3).

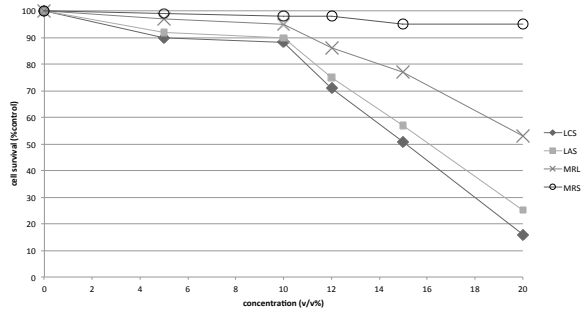

Figure 1. Cell Growth Inhibitory Effects of Different Concentrations of Lactobacillus Acidophilus Culture Supernatant (LAS), Lactobacillus Crispatus Culture Supernatant (LCS), MRS and MRL on MDA-MB-231 Cells

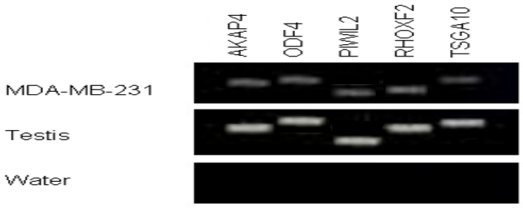

Figure 2. Expression of 5 Cancer-testis Antigens in MDA-MB-231 Cell Line and Testis Sample

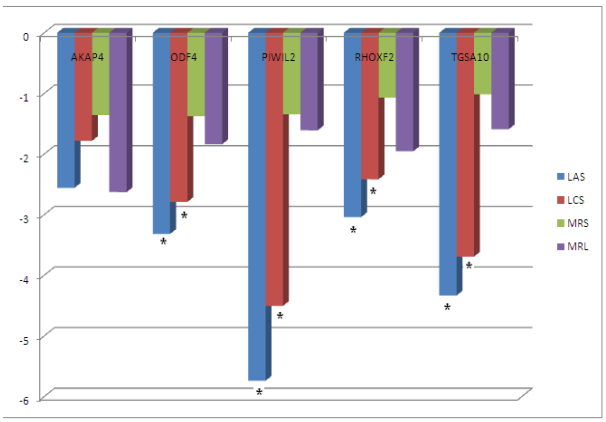

Figure 3. The Effects of Lactobacillus Acidophilus Culture Supernatant (LAS), Lactobacillus Crispatus Culture Supernatant (LCS), MRS and MRL on Expression of 5 Cancer-testis Antigens. Asterisks indicate $p<0.05$

Discussion

Antitumor activities of probiotic bacteria have been demonstrated previously (Russo et al., 2007). However, the mechanism of their antiproliferative function is not fully understood. Different components of lactobacilli could exert this effect. For instance, it has been shown that cytoplasm extracts but not cell-wall fractions are responsible for antitumor effects of *Lactobacillus rhamnosus* strain GG on HGC-27 cell (Russo et al., 2007; Orlando et al., 2012). Besides, cell-bound exopolysaccharide from *Lactobacillus acidophilus* has shown antitumor activities against colon cancer cells (Kim et al., 2010). In a previous study aimed to compare the antiproliferative effects of the supernatants, cytoplasmic extracts, cell-wall extracts and live lactobacilli on normal and tumor cervical cell lines, we have shown that culture supernatants of lactobacilli have the most effective cytotoxic functions (Motevaseli et al., 2013a). Consequently, in this study we have assessed the effects of culture supernatants of two lactobacilli on expression of 5 CTAs known as cancer biomarkers and have shown that their culture supernatants can significantly decrease transcriptional activity of most of them. Previously, it has been demonstrated that oral administration of *Lactobacillus acidophilus* can shift the cytokine response in tumor bearing mice into a Th1 protective pattern, beneficial to anti tumor immunity. In addition, decreased tumor growth rate and enhanced lymphocyte proliferation were also demonstrated (Maroof et al., 2012). As both *Lactobacillus Acidophilus* and *Lactobacillus Crispatus* are oral probiotics, administration of them in tumor-bearing patients can be of benefit.

AKAP4 is a scaffolding protein and binds to cAMP dependent Protein Kinase A (PKA) (Gold et al., 2006) which has been suggested to be involved in most of human tumors and malignant features such as cell proliferation, angiogenesis, and chemoresistance (Saini et al., 2013b). A recent study has shown that AKAP4 silencing significantly inhibits cellular proliferation, colony-forming ability, migration and invasion ability of cervical cancer cells as well as tumor growth in nude mice in vivo (Saini et al., 2013a). Although LAS could decrease transcriptional activity of AKAP4, this effect was not statistically significant when compared to MRL. This result implies that the transcriptional downregulation of this gene can be attributed to the acidity of culture supernatant.

The main role of Odf proteins is to maintain sperm tail. However, some of them have been shown to be components of the centrosome matrix (Nakagawa et al., 2001). Considering the essential role of centrosome in efficient mitosis, overexpression of ODF genes in cancer cells may be involved in their rapid proliferation. In this study, we demonstrated that both culture supernatants could decrease transcriptional activity of ODF4 in MDA-MB-231 cell. Future researches should focus on the effects of ODF downregulation on cell mitosis and proliferation.

PIWIL2 is shown to be involved in RNA silencing and

self-renewal. In a study of colorectal cancer cases, it has been revealed that the high degree of PIWIL2 expression is correlated with the lower degree of differentiation, higher rate of invasion and shorter survival. So it has been concluded that PIWIL2-positive cells contribute to the progression of colorectal cancer. It has been suggested that PIWIL2 plays a role in regulation of the expression of genes related to proliferation and apoptosis through small RNA and methylation. Its role as an oncogene inhibiting apoptosis and promoting proliferation via STAT3/Bcl-XL signaling has also been demonstrated (Oh et al., 2012). Our result show that both examined lactobacilli can significantly decrease the expression of PIWIL2 in MDA-MB-231 cells which could have therapeutic implications.

RHOXF2 codes for a homeobox protein which is a transcription factor functioning in embryonic, postnatal and adult development. Epigenetic mechanisms have been shown to be involved in its transcriptional regulation. Its knockdown has reduced the growth of a gastric cancer cell line while its overexpression in HF6 cells has rapidly induced leukemia in transplanted mice (Shibata-Minoshima et al., 2012). As RHOXF2 is involved in cell transformation, its downregulation by lactobacilli may have clinical implications for cancer treatment.

TSGA10 is CTA shown to be expressed in a variety of tumor samples including breast cancer (Mobasheri et al., 2006; 2007; Dianatpour et al., 2012). Although it has been suggested as a potential oncogene in many cancers, recently it has been shown that it acts a tumor suppressor gene in esophageal squamous cell carcinoma (ESCC) regulating G1-S transition (Yuan et al., 2013). However, as proposed by the authors, this pathway may be restricted to ESCC. Here, we showed that lactobacilli could decrease transcriptional activity of TSGA10 in MDA-MB-231.

Epigenetic mechanisms, principally DNA methylation, have been suggested as major regulators of CTA gene expression in normal and cancer cells in addition to stem cells. Combination of epigenetic modulatory drugs and CTA immunotherapy has been suggested as a therapeutic approach for cancer treatment (Akers et al., 2010). This study implies that lactobacilli can exert their anticancer effects via epigenetic regulation of CTAs. Further researches are needed to evaluate the effects of CTA downregulation on cell proliferation, invasion and metastasis. In addition, the antiproliferative effects of lactobacilli should be evaluated in other cancer cell lines, which would open a new era for clinical applications.

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