

Aim of the study: *Lactobacillus casei* (*L. casei*) has been shown to inhibit the proliferation of several types of cancer *in vivo*, but its effect on cervical cells has not been reported. We incubated cells of the human cervical cell lines Caski and HeLa with extracts of *L. casei* and investigated its effects on the growth of the cells and possible synergy with anticancer drugs.

Material and methods: Cell-free extracts of *L. casei* were prepared and purified. Cultures of Caski and HeLa cells adhering to tissue culture plates were treated with *L. casei* extract. The effects of *L. casei* extract on the growth of cancer cells and its possible synergy with anti-cancer drugs in cervical cancer cell lines were investigated. The cells were treated with *L. casei* extract alone, anti-cancer drugs alone [doxorubicin, paclitaxel, 5-fluorouracil (5-FU), and cisplatin], or *L. casei* extract plus anti-cancer drugs.

Results: *L. casei* extract had no significant effect on the growth rate of the two cell lines. Anti-cancer drugs alone induced growth inhibition, but there was no synergistic effect of *L. casei* extract on growth inhibition.

Conclusions: *L. casei* extract does not have a potent effect on the viability of cervical cancer cells *in vitro*. In addition, *L. casei* extract has no synergistic effect on the inhibition of growth of cancer cells in the presence of anti-cancer drugs.

Key words: *Lactobacillus casei*, cell viability, apoptosis, chemotherapy.

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The effect of *Lactobacillus casei* extract on cervical cancer cell lines

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Introduction

Lactobacillus casei (*L. casei*) is a Gram-positive, non-pathogenic bacterium, found mainly in the human intestine [1]. It produces lactic acid as a fermentation product of carbohydrates [2] and is used as an acid-producing starter culture in the preparation of fermented foods [3]. As a nutritional supplement, *L. casei* has been shown to lead to improvements in intestinal microbial balance [4], arthritis [5], and type 1 diabetes [6]. It has also been shown to have anti-cancer effects via growth inhibition in leukaemia [7] and liver cancer [8]. Many female cancer patients take *L. casei* as a nutritional supplement.

Cervical cancer is the second leading cause of cancer mortality among women worldwide. In Korea, in the past, cervical cancer had the highest incidence, but in recent years thyroid cancer and breast cancer have overtaken it. According to Korean Central Cancer Registry Project Statistics, cervical cancer accounted for one-eighth of all carcinomas in Korean women in 2009 [9]. The frequency of cervical cancer is gradually falling, largely because regular cancer screening prevents dysplasia and carcinoma *in situ* from progressing to invasive cancer. However, cervical cancers occupy an important position among all cancers if carcinoma *in situ* and dysplasia are included. Cervical cancer is associated with viral infection [10]; the most important cause of cervical cancer is persistent cervical infection with human papillomavirus (HPV) [11]. Bacterial vaginosis (BV) is caused by an alteration in the vaginal flora involving a decrease in *Lactobacilli* and predominance of anaerobic bacteria. A meta-analysis has shown that BV is associated with uterine cervical HPV infection [12]. Development of vaccines for the prevention and treatment of cervical cancer is ongoing.

Traditional treatment methods for women's cancer are surgery, radiation and chemotherapy. Surgery is carried out in cases with no metastasis to other organs, whereas chemotherapy and radiation are performed after surgery or in cases where there is metastasis to other organs. Many female patients take nutritional supplements, such as anti-oxidant agents, fish oil, and *L. casei*, before, during, and after cancer therapy [13]. The effect of these nutritional supplements depends on the cancer type and the patient's lifestyle. However, there are few studies of the effects of anti-cancer drugs plus nutritional supplements in cancer. The aim of the current study was to examine the effect of *L. casei* extract on growth inhibition in cervical cancer cells.

Material and methods

Bacteria strains and growth conditions

Lactobacillus casei ATCC393 (American Type Culture Collection) was obtained from the Korean Culture Centre of Microorganisms (KCCM). The bacteria were grown in *Lactobacillus* Mann-Rogosa-Sharp (MRS) broth (Difco

0881) at 37°C. The growth medium consisted of 10 g Difco proteose peptone No. 2, 10 g beef extract, 5 g yeast extract, 20 g glucose, 1 g sorbitan monooleate complex (Tween 80), 5 g ammonium acetate, 5 g sodium acetate, 2 g K_2HPO_4 , 1 g $MgSO_4 \cdot 7H_2O$, and 0.05 g $MnSO_4 \cdot 4H_2O$. The pH of the growth medium was adjusted to 6.5 after mixing the components.

Preparation and purification of cell-free extracts of *Lactobacillus casei*

Lactobacillus casei was grown in MRS medium for two days until the mid-exponential phase ($A_{600} = 0.5$), then it was centrifuged and re-suspended in 500 μ l 1 × phosphate-buffered saline (PBS) with two further washes, and disrupted on ice for 1 minute with a Sonic Dismembrator at 5 Watts. The extract was centrifuged and the supernatant

filtered through a 0.2 μ m syringe filter. The protein concentration of the extract was assayed by the Bradford method, with bovine serum albumin (BSA) as the standard.

Cell culture

CaSki and HeLa cell lines were obtained from the Korean Cell Line Bank (Seoul, South Korea). The cells were cultured on tissue culture dishes (Falcon; San Jose, CA, USA) in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS). Aliquots of 5×10^5 cells/dish were cultured at 37°C in a humidified atmosphere containing 5% CO_2 .

Cell growth rate measurement

For each cell line, 5×10^5 cells were seeded in DMEM containing 10% FBS. After 24 hours the cells were washed

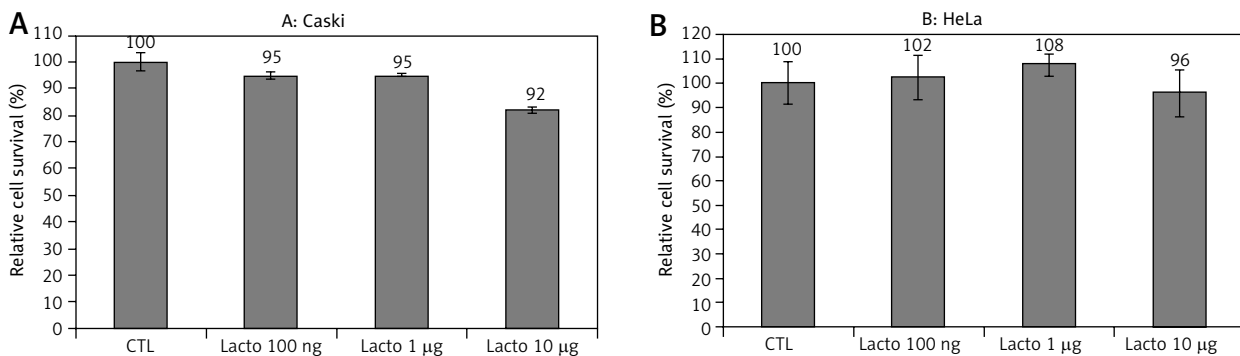


Fig. 1. Effect of *L. casei* extract on cervical cancer cell lines. **A)** Caski and **B)** HeLa cells were treated with extract of *L. casei* at 1, 10, or 100 M. Cells were stained with trypan blue to determine viability. Viable cell numbers in the treated cultures are shown relative to the numbers in the controls. The data are means of at least three independent experiments with the corresponding standard errors

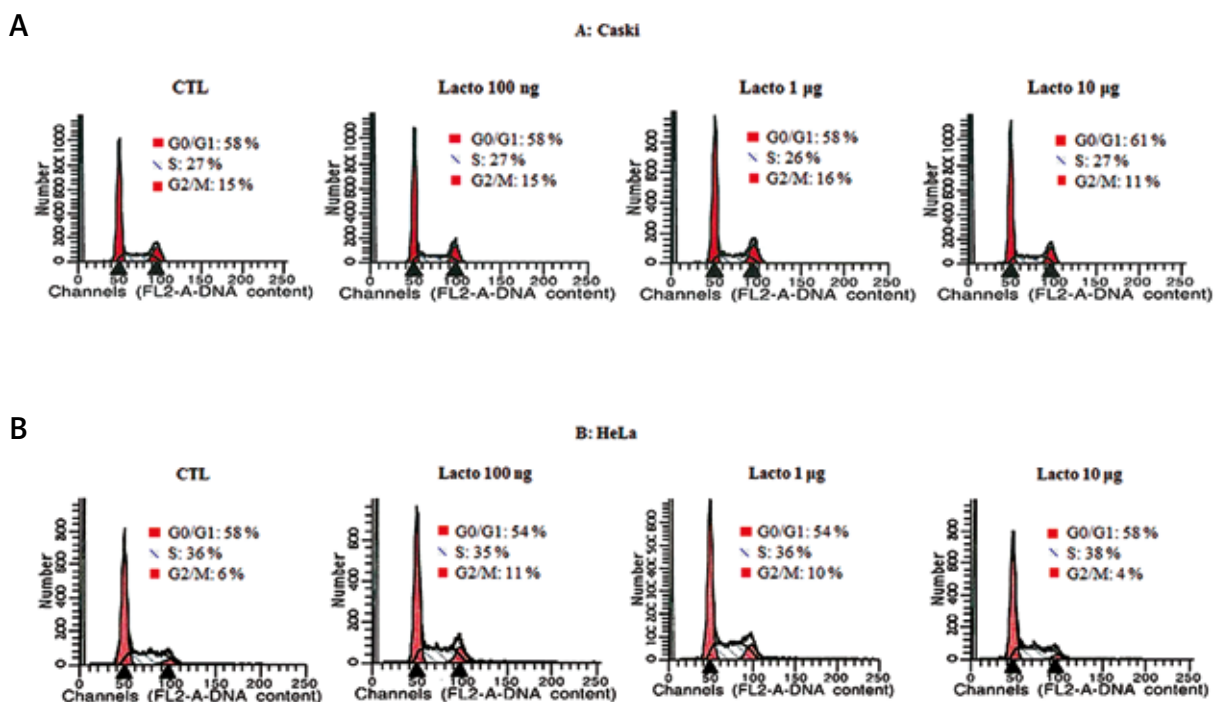


Fig. 2. *Lactobacillus casei* treatment does not affect cell cycle phases. **A)** Caski cells and **B)** HeLa cells were treated with the indicated amounts of the extract of *L. casei* for 72 hours. The percentages of cells at each stage of the cell cycle were analysed by flow cytometry after DNA staining with propidium iodide. The data are from a representative experiment

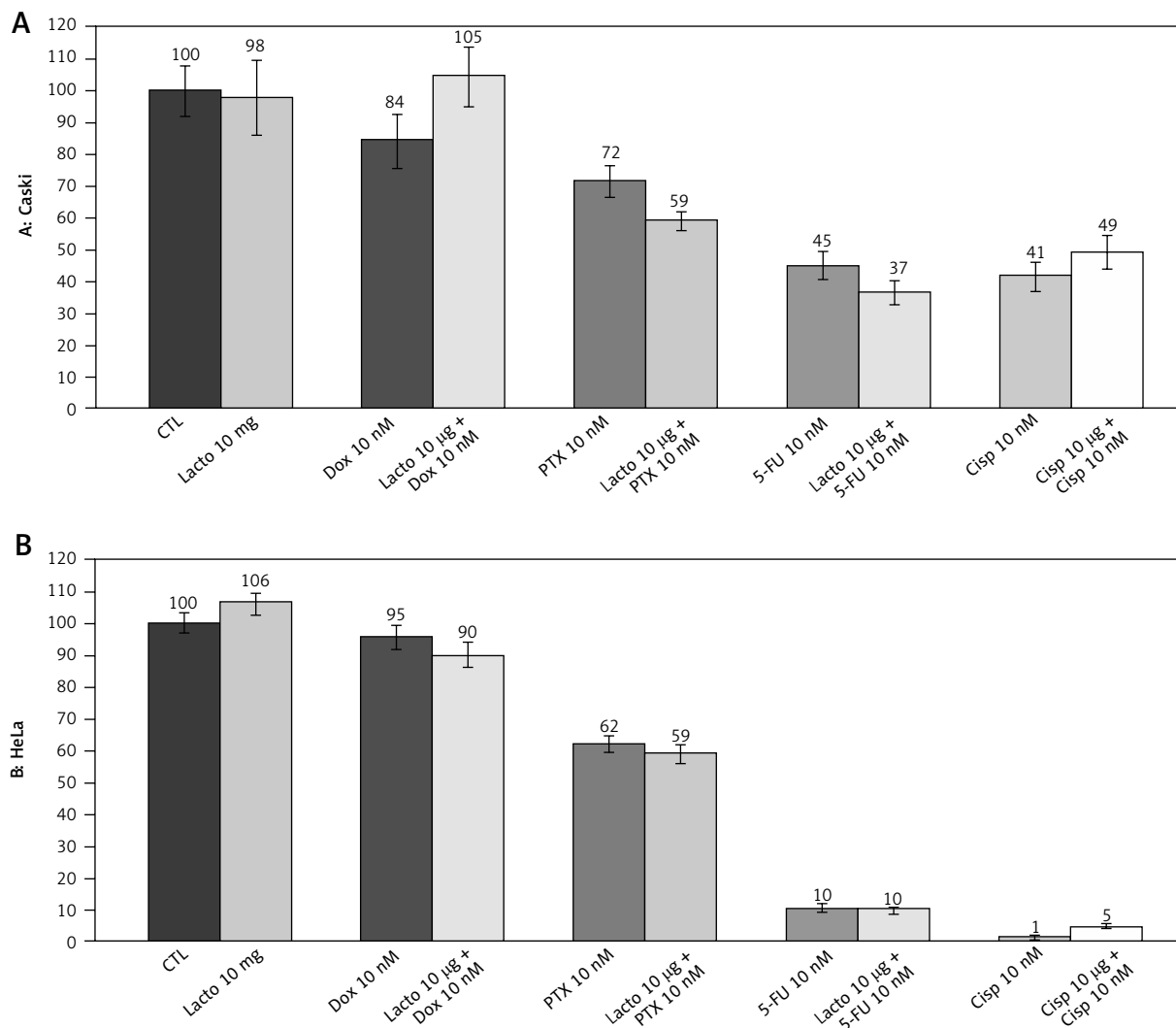


Fig. 3. Growth rate of CaSki and HeLa cells after treatment with *Lactobacillus casei* extract and anti-cancer drugs. **A)** CaSki cells. **B)** HeLa cells. Aliquots of 5×10^5 cells were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS). Cells were treated with distilled water, followed by *L. casei* extract (10 µg/ml), anti-cancer drugs (doxorubicin 10 nM, 5-fluorouracil (5-FU) 10 nM, paclitaxel 10 nM, cisplatin 10 nM) or *L. casei* extract (10 µg/ml) plus anti-cancer drugs (doxorubicin 10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM) for 72 hours. Cells were then stained with trypan blue to determine viability. Viable cell numbers in the treated cultures are shown relative to controls

with PBS and cultured in fresh medium. Cells were treated with distilled water, followed by 100 ng, 1 µg, or 10 µg/ml *L. casei* extract alone, anti-cancer drugs alone (doxorubicin 10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM), or *L. casei* extract (10 µg/ml) plus anti-cancer drugs (doxorubicin 10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM) for 72 hours. The growth rate was determined using an inverted microscope (Olympus Model IX51, Tokyo, Japan) equipped with a DP50 camera system (Olympus, Tokyo, Japan) using the 0.4% trypan blue dye exclusion method. Cell survival was measured as a percentage of the total number of cells (viable plus non-viable). Mean survival was determined by counting four randomly selected non-overlapping fields. Each culture dish represents one determination, with each experiment replicated independently 4–6 times using different cultures.

Cell cycle analysis

CaSki and HeLa cells were seeded at a density of 1×10^5 cells/ml in DMEM medium containing 10% FBS. After 24 hours they were incubated with distilled water, *L. casei* extract (10 µg/ml), or *L. casei* extract (10 µg/ml) plus anti-cancer drugs (doxorubicin 10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM) for 72 hours. The cells were then incubated with 100 µg/ml RNase A for 1 hour at 37°C, and stained with 10 µg/ml propidium iodide (PI). Flow cytometry was performed in triplicate for each experiment using an FACS Calibur system (BD Biosciences, San Jose, CA, USA).

Analysis of apoptosis by annexin V staining

CaSki and HeLa cells were seeded at a density of 1×10^5 cells/ml in DMEM medium containing 10% FBS. After

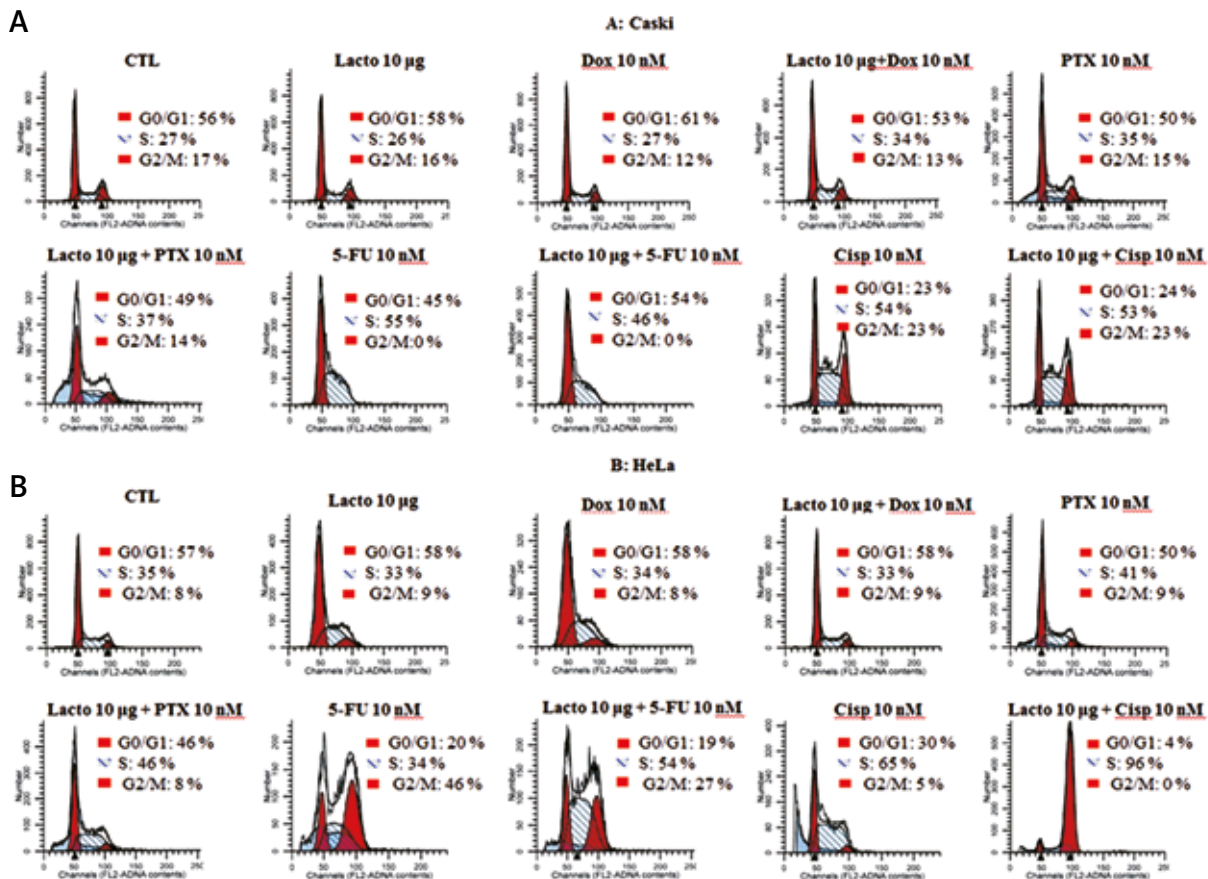


Fig. 4. Cell cycle analysis for CaSki and HeLa cells after treatment with *L. casei* extract and anti-cancer drugs. **A)** CaSki cells. **B)** HeLa cells. Aliquots of 5×10^5 cells were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS). Cells were treated with distilled water, followed by *L. casei* extract (10 µg/ml), anti-cancer drugs (doxorubicin 10 nM, 5-fluoruracil (5-FU) 10 nM, paclitaxel 10 nM, cisplatin 10 nM) or *L. casei* extract (10 µg/ml) plus anti-cancer drugs (doxorubicin 10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM) for 72 hours. The percentages of cells in each stage of the cell cycle were analysed by flow cytometry

24 hours, they were incubated with distilled water, *L. casei* extract (10 µg/ml) or *L. casei* extract (10 µg/ml) plus anti-cancer drugs (doxorubicin 10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM) for 72 hours. Annexin V staining was performed in accordance with the manufacturer's protocol (BD Pharmingen, NJ, USA). Trypsin-EDTA was used to obtain single-cell suspensions. After centrifugation, the cells were washed twice with cold PBS and re-suspended in a binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂ and 1.8 mM CaCl₂) at a concentration of 1×10^6 cells/ml. An aliquot (100 µl) containing 1×10^5 cells was transferred to a 5 ml culture tube, and 5 µl each of Annexin V-FITC and PI were added. After vortexing the tubes, the cells were incubated for 15 minutes at room temperature (25°C) in the dark and 400 µl of binding buffer was added to each tube. Flow cytometry was performed with the FACS Calibur system within 1 hour.

Statistical analysis

All data were compiled from a minimum of three replicate experiments and expressed as means \pm standard errors. Student's *t*-test was used to compare treated cells with control cells. A *p*-value less than 0.05 was considered statistically significant.

Results

Effect of *Lactobacillus casei* extract on the growth rate of cervical cancer cells

To observe the effects of *L. casei* extract on cancer cell growth, two cell lines (CaSki, HeLa) were treated with the extract at several concentrations (Fig. 1). There was evidence of growth inhibition at 10 µg, but the effect was not statistically significant.

Analysis of the cell cycle in *Lactobacillus casei*-treated cells

The results of cell cycle analysis showed a tendency towards cell cycle changes, but they were again not statistically significant (Fig. 2). In cell cycle analysis by PI staining, there was a 4% increase in the DNA content of CaSki cells in the G0/G1 phase (Fig. 2A), whereas the HeLa cells showed a 2% increase in DNA content in the S phase (Fig. 2B).

Effect of *Lactobacillus casei* extract and anti-cancer drugs on growth of CaSki and HeLa cells

To measure any enhanced effect of *L. casei* extract plus anti-cancer drugs, CaSki and HeLa cells were treated with *L. casei* extract alone, anti-cancer drugs alone (doxorubicin

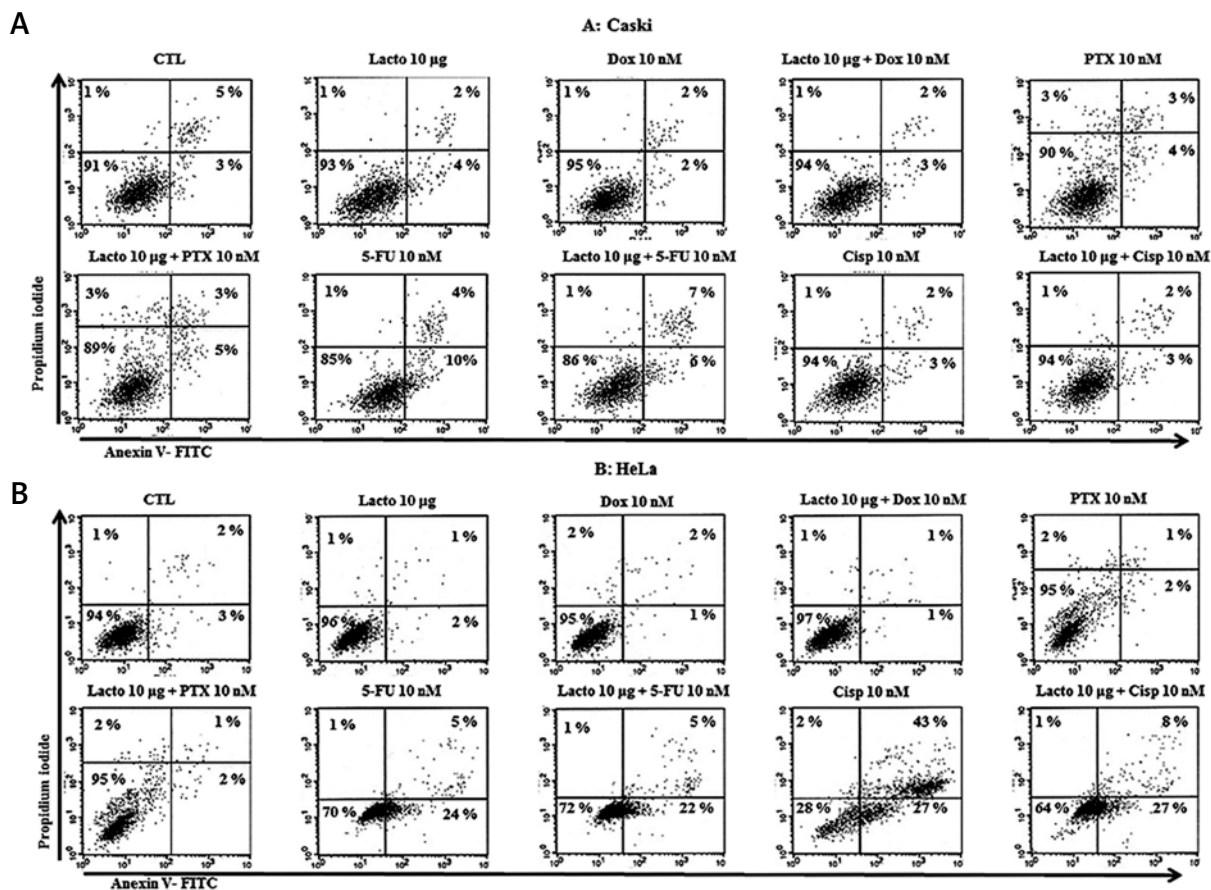


Fig. 5. Cell death analysis after treatment of CaSki and HeLa cells with *L. casei* extract and anti-cancer drugs. **A)** CaSki cells. **B)** HeLa cells. Aliquots of 5×10^5 cells were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS). Cells were treated with distilled water, followed by *L. casei* extract (10 μ g/ml), anti-cancer drugs (doxorubicin 10 nM, 5-fluoruracil (5-FU) 10 nM, paclitaxel 10 nM, cisplatin 10 nM), or *L. casei* extract (10 μ g/ml) plus anti-cancer drugs (doxorubicin 10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM) for 72 hours. Cell death was analysed by annexin V staining using flow cytometry. For each treatment, the percentage of viable cells is shown in the lower left quadrant

10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM), or *L. casei* extract plus anti-cancer drugs (doxorubicin 10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM). All of the anti-cancer drugs inhibited the growth of these cells, but *L. casei* extract had no additional effect (Fig. 3A, B).

Effect of *Lactobacillus casei* extract and anti-cancer drugs on the cell cycle in CaSki and HeLa cells

The cell cycle is associated with growth rate in cancers. The effect of *L. casei* extract plus anti-cancer drugs on the cell cycle was studied by growing the cells as above. The cell cycles of both CaSki and HeLa cells were affected in response to *L. casei* extract. There was a 7% increase in S phase cells when CaSki cells were exposed to doxorubicin and a 10% increase when the cells were exposed to 5-FU plus *L. casei* extract (Fig. 4A). Similarly there was a 5% increase in S phase cells when HeLa cells were incubated with paclitaxel, a 20% increase with 5-FU, and a 31% increase when treated with cisplatin plus *L. casei* extract (Fig. 4B). Thus, *L. casei* extract is potently associated with cell cycle arrest, but has no synergistic effect on growth inhibition in cervical cancer cells.

Effects of *Lactobacillus casei* extract and anti-cancer drugs on cell death in CaSki and HeLa cells

The effects of chemotherapeutic drugs on CaSki and HeLa cells were investigated by measuring apoptosis using annexin V staining. There was no synergistic effect on cell death when CaSki cells were treated with *L. casei* extract and anti-cancer drugs (doxorubicin 10 nM, 5-FU 10 nM, paclitaxel 10 nM, cisplatin 10 nM) (Fig. 5A). There was also no enhanced effect on cell death in HeLa cells when exposed to *L. casei* extract and the following anti-cancer drugs: doxorubicin 10 nM, 5-FU 10 nM, and paclitaxel 10 nM. However, when HeLa cells were treated with *L. casei* extract and cisplatin, there was a 36% increase in survival compared with other anti-cancer drugs (Fig. 5B).

Discussion

Several strains of *Lactobacillus* have potent anti-tumour effects in rodents [14]. *Lactobacillus casei* potentiates systemic immune responses in mouse models of colon cancer [15] and reduces tumour induction when given orally [16]. *Lactobacillus casei* potentiates systemic immune responses that modify T-cell functions in mice and induces

a cellular immune response that inhibits tumour growth after peritoneal inoculation [17]. Orally administered *L. casei* has an inhibitory effect on the recurrence of superficial bladder cancer [18]. These results indicate that *L. casei* may have anti-cancer effects in humans. However, the anti-tumour activity of *L. casei* on cervical cancer has not been reported previously.

This study examined the effect of *L. casei* extract on the following cervical cancer cell lines (CaSki, HeLa). The mechanism of anti-cancer activity of *L. casei* is not known. Soluble polysaccharide components from *Lactobacilli* strains exhibit inhibitory activity in cancer cell lines [19] and a cell-wall-derived peptidoglycan has anti-cancer activity [20]. We therefore used whole extract of *L. casei*. In this study, the extract did not affect the growth of women's cancer cell lines, and there was no synergistic effect after concomitant administration of one or more chemotherapeutic drugs. Instead, we found that *L. casei* extract mainly induced enhanced S-phase cell cycle arrest in cervical cancer cells when administered together with anti-cancer drugs other than 5-FU. In contrast to the effect on the cell cycle, one chemotherapeutic drug (cisplatin) had an inhibitory effect on apoptosis when administered together with *L. casei* extract. Thus, *L. casei* extract may have an anti-cancer effect in cervical cancer through an effect on cell cycle arrest, although it has no effect on growth inhibition or any synergistic effect on women's cancer cell growth when administered together with a chemotherapeutic drug.

The cell cycle is the most important factor involved in cancer progression and growth [21]. Molecules involved in the cell cycle have therefore been targeted to inhibit the growth rate of several types of cancer. With respect to cell cycle arrest in cancer growth, we reported previously that *L. casei* extract arrested the cell cycle and induced growth inhibition in liver cancer [8]. Matsuki *et al.* reported a direct effect of *L. casei* on growth inhibition [22]. In a co-culture system of *L. casei* with intestinal epithelial cells, they showed that *L. casei* down-regulated cell cycle expression of cyclin E1 and D1, and caused growth inhibition [22]. These results indicate that *L. casei* has an effect on growth inhibition through cell cycle arrest.

In contrast to its effect on the cell cycle, cisplatin inhibited cell death when administered together with *L. casei* extract. This may result from a high-dose effect on cell growth. In our study, cisplatin induced more than 90% inhibition of growth in HeLa cells. However, although this result is significant, it may result from experimental error; it is possible that the effect of *L. casei* extract on cell death may be the result of using an inappropriate dose of cisplatin. Therefore, the effect of *L. casei* extract on inhibition of cell death requires further examination by several different methods.

A limitation of this study was that we applied *L. casei* extract directly to the cancer cells *in vitro*, since there is no objective *in vivo* means of measuring *L. casei* absorption into the human body. Further studies of immunologic responses, other pathways of growth inhibition, *in vivo* effects, and physical changes due to long-term intake of *L. casei* extract are required in *in vivo* cancer models.

In conclusion, direct treatment with *L. casei* extract has no effect on the growth of cervical cancer cell lines CaSki and HeLa, and it has no synergistic effect with the chemotherapeutic agents tested in this study.

The authors declare no conflict of interest.

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