Normal and tumour cervical cells respond differently to vaginal lactobacilli, independent of pH and lactate

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Cervical cancer is a human papilloma virus (HPV)-related cancer, but most HPV infections are transient or intermittent and resolve spontaneously. Thus, other factors, such as cervical microflora, which are dominated by lactobacilli, must be involved in invasive cervical carcinoma development after HPV infection. Previous studies have demonstrated that lactobacilli have antitumour effects, and it is possible that vaginal lactobacilli prevent cervical cancer. Here we examined the proliferative and apoptotic responses of normal and tumour cervical cells to common vaginal lactobacilli components by investigating human normal fibroblast-like cervical (normal cervical) and HeLa (cervical tumour) cell responses to Lactobacillus gasseri and Lactobacillus crispatus. The effects of different lactobacilli components, such as culture supernatants, cytoplasmic extracts, cell-wall extracts and live cells, were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, trypan blue staining, lactate dehydrogenase assay and colorimetric caspase-3 activity assay. Changes in caspase-3 and human chorionic gonadotropin (hCGβ) expression were analysed by quantitative RT-PCR.

Tumour cell growth inhibition by culture supernatants was higher than that by pH- and lactate-adjusted controls. However, the effects of the supernatants on normal cells were similar to those of lactate-adjusted controls. Apoptosis was inhibited by supernatants, which was consistent with higher hCGβ expression since hCG inhibits apoptosis. Our study demonstrated that common vaginal lactobacilli exert cytotoxic effects on cervical tumour cells, but not on normal cells, and that this cytotoxicity is independent of pH and lactate. Our results encourage further studies on the interaction between lactobacilli and cervical cells, and administration of common vaginal lactobacilli as probiotics.

INTRODUCTION

Cervical cancer is the most frequently diagnosed cancer among women in developing countries, and the second most frequent cancer affecting women worldwide (Sankaranarayanan & Ferlay, 2006). It progresses through a series of premalignant stages – cervical intraepithelial neoplasia (CIN) 1, 2 and 3 (Apgar et al., 2003). It takes 10–20 years for a normal cervical epithelial cell to become malignant, and only some women with CIN develop invasive cancer. The most well-known risk factor for this cancer is human papilloma virus (HPV) infection, but despite the high prevalence of HPV infection, CIN incidence and progression rates of untreated CIN lesions are low (Nam et al., 2009). Over 90% of HPV infections and infection-induced lesions are transient or intermittent and resolve spontaneously (Bosch et al., 2002). Although HPV infection plays a major role in cervical cancer pathogenesis, it alone is insufficient, and other environmental and host factors, such as the existing cervical microbial flora and infections, are probably associated with disease development.
Four species of lactobacilli are predominantly associated with the reproductive and general health of women (Tamrakar et al., 2007; Li et al., 2007; Ravel et al., 2011; Xiao & Liao, 2012; Pendharkar et al., 2013). Some lactobacilli (such as *Lactobacillus crispatus* and *Lactobacillus gasseri*) are probiotics (Isolauri, 2001; Yan & Polk, 2002; Ngugi et al., 2011; Fujimura et al., 2012). Probiotic bacteria control the overgrowth and infectious processes of pathogens (Johnson-Henry et al., 2003; Hemarajata & Versalovic, 2013), cell proliferation (Orlando et al., 2009, 2012), and apoptosis (Iyer et al., 2008; Li et al., 2011; Sharma et al., 2011). *Lactobacillus rhamnosus* GG prevents cytokine-induced apoptosis in intestinal epithelial cells (Yan & Polk, 2002). Metabolic products of lactobacilli, such as hydrogen peroxide, lactate and bacteriocins, also play an important role in the maintenance of the normal vaginal flora by inhibiting colonization by other pathogens (Tamrakar et al., 2007; Spurbeck & Arvidson, 2011). These microorganisms prevent colonization of the urogenital tract by pathogens, and are important for the reproductive and general health of women (Tamrakar et al., 2007; Li et al., 2012).

Four species of lactobacilli are predominantly associated with vaginal microfiora: *L. crispatus*, *Lactobacillus jensenii*, *L. gasseri* and *Lactobacillus iners* (Ravel et al., 2011). The cervix of healthy women is known to be mostly colonized with lactobacilli. Lactobacilli have an essential role in maintaining homeostasis of the vagina by preventing the growth of pathogenic micro-organisms and inhibiting yeast infections (such as vaginal candidiasis) and bacterial vaginosis (BV) (Ravel et al., 2011). In fact, reduction of lactobacilli prepares conditions for other micro-organisms to grow (Martin et al., 2008). Women with BV lose most *Lactobacillus* species, and instead acquire several anaerobic and facultative bacteria (Zozaya-Hinchcliffe et al., 2010). Some reports have confirmed a significant correlation between BV and CIN (Nam et al., 2009). These data and the probiotic properties of vaginal lactobacilli indicate their relationship with cervical cancer and their effects on its characteristic features such as human chorionic gonadotropin (hCG) secretion.

hCG, composed of two noncovalently linked subunits α (hCGα) and β (hCGβ), is physiologically produced by the placenta (Gharib et al., 1990). However, tumours with different origins secrete hCG (Iles, 2007). For example, sera and urine samples from patients with nonthrophoblastic cancers (e.g. cervix, ovary, gastrointestinal tract, bladder, lung and prostate cancers) have shown hCGβ immuno-reactivity (Jankowska et al., 2008a). Recent studies have shown that hCGβ (mRNA and protein) is a characteristic feature of cervical carcinomas and acts as an autocrine growth factor by inhibiting apoptosis (Jankowska et al., 2008b). Elevated serum hCGβ levels correlate with increased cancer aggressiveness and its resistance to therapy (Jankowska et al., 2008b).

The effects of vaginal lactobacilli on cervical cells remain unelucidated. We aimed to evaluate the proliferative and apoptotic responses of normal and tumour cervical cell lines to different components of two common vaginal lactobacilli (*L. crispatus* and *L. gasseri*) and evaluate the changes in hCGβ expression in cervical cells.

**METHODS**

**Preparation of lactobacilli components**

**Preparation of supernatants from Lactobacillus cultures.** *L. crispatus* strain SJ-3C-US (LbC) and *L. gasseri* ATCC 33323 (LbG) were grown in de Man Rogosa Sharpe (MRS) broth (Merck; pH 6.5) at 37°C for 24 h under microaerophilic conditions. MRS contains a rich nutrient base, polysorbate, acetate, magnesium and manganese, which are known to promote the growth and proliferation of lactobacilli. Overnight bacterial cultures contained 2 x 10⁶ c.f.u. ml⁻¹, and these cultures were centrifuged at 1100 g for 15 min at 4°C. The resulting lactobacilli supernatants (LS) were filtered through a 0.2 μm membrane filter to remove the remaining bacteria and debris. The pH of the LS was 4.0 ± 0.1, and decreased from 6.5 (MRS broth pH). To determine whether lactate produced by the two lactobacilli and pH change would affect tests, the lactate concentration in LS was determined using a Lactate Randox kit (Randox Laboratories); noncultured MRS broth adjusted to pH 4 with HCl and/or lactate was used in co-culture tests. In some experiments, the pH of the LS was adjusted to 6.5 with NaOH for pH control. The following were tested: LbG supernatant, pH 4 (LGS); LbC supernatant, pH 4 (LCS); LbG supernatant, pH 6.5 (LGSN); LbC supernatant, pH 6.5 (LCSN); MRS pH 4, adjusted with HCl (MRH); MRS pH 4, adjusted with lactate (MRL).

**Preparation of lactobacilli-conditioned media and live lactobacilli.** *L. crispatus* strain SJ-3C-US and *L. gasseri* ATCC 33323 were grown in MRS broth (pH 6.5) at 37°C for 24 h under microaerophilic conditions. Overnight bacterial cultures contained 2 x 10⁶ c.f.u. ml⁻¹. Bacterial cultures were harvested by centrifugation at 1100 g for 15 min. Supernatants were discarded, and bacterial pellets were washed twice in PBS (pH 7.4) and resuspended in HeLa cell medium (RPMI, 10% fetal calf serum, 1.5% HEPES, pH 7.2) without antibiotics. For live bacterial assays, different m.o.i. of lactobacilli were used. Conditioned media were prepared as described by Yan & Polk (2002). After 2 h suspension in RPMI at 37°C in 5% CO₂, cultures were centrifuged at 1000 g and supernatants recovered were filtered through a 0.2 μm membrane filter. The following were tested: LbG supernatant in RPMI, pH 6 (LGRS); LbC supernatant, pH 6 (LCRS); LbG supernatant in RPMI, adjusted with NaOH, pH 7.2 (LGRN); LbC supernatant in RPMI, adjusted with NaOH, pH 7.2 (LCRN); RPMI with lactate, pH 6 (RPMIL); RPMI adjusted with HCl, pH 6 (RPMH); RPMI, pH 7.2.

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**Table 1. Oligonucleotide sequences of the forward (F) and reverse (R) primers, and the probe (P)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’→3’ )</th>
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<tr>
<td>hCGβ</td>
<td>F – GCTACTGCCCCACCATGACC&lt;br&gt;R – ATGGACTGAGGCCACATC&lt;br&gt;P – CCTGCTCAGGGTGTTGCAACTACC</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>F – AATTGTGGAATTGCGTGATG&lt;br&gt;R – CTACACGATCCTCCTTGA AAA</td>
</tr>
<tr>
<td>HPRT</td>
<td>F – CCTGGGCTGTGGATTAGTGAT&lt;br&gt;R – AGACGTTCA GTCTGTCCATAA</td>
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The healthy human vaginal and cervical ecosystem is dominated by lactobacilli (Vasquez et al., 2002; Liu et al., 2007; Ravel et al., 2011; Xiao & Liao, 2012; Pendharkar et al., 2013). Some lactobacilli (such as *Lactobacillus crispatus* and *Lactobacillus gasseri*) are probiotics (Isolauri, 2001; Yan & Polk, 2002; Ngugi et al., 2011; Fujimura et al., 2012). Probiotic bacteria control the overgrowth and infectious processes of pathogens (Johnson-Henry et al., 2003; Hemarajata & Versalovic, 2013), cell proliferation (Orlando et al., 2009, 2012), and apoptosis (Iyer et al., 2008; Li et al., 2011; Sharma et al., 2011). *Lactobacillus rhamnosus* GG prevents cytokine-induced apoptosis in intestinal epithelial cells (Yan & Polk, 2002). Metabolic products of lactobacilli, such as hydrogen peroxide, lactate and bacteriocins, also play an important role in the maintenance of the normal vaginal flora by inhibiting colonization by other pathogens (Tamrakar et al., 2007; Spurbeck & Arvidson, 2011). These microorganisms prevent colonization of the urogenital tract by pathogens, and are important for the reproductive and general health of women (Tamrakar et al., 2007; Li et al., 2012).

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Preparation of homogenates, cytoplasmic extracts and cell-wall extracts. We prepared homogenates, cytoplasmic extracts and cell-wall extracts of lactobacilli as described by Russo et al. (2007). Precipitates from lactobacilli cultures were washed twice with PBS (pH 7.4); then bacterial cells were disrupted by sonication with an Ultrasonic 1000 sonicator (Hielscher) on ice at 50 W for 1 min at 30 s intervals. Cell number was calculated using light microscopy, and the number of ruptured cells was determined by counting intact cells before and after sonication. Sonicated bacteria were suspended in PBS to a final concentration of 10^8 cells ml^{-1}. Finally, preparations were centrifuged at 1000 g for 30 min at 4 °C, and supernatants were used as homogenates and stored at -70 °C until use. To obtain cell-wall extracts from the pellet fraction and cytoplasmic extracts from the supernatant fraction, aliquots of the homogenates were ultracentrifuged at 35 000 g for 20 min at 4 °C. Cell-wall extracts were prepared from the pellet by suspension in PBS so that an amount equal to that of the supernatant was obtained. All preparations were filtered through a 0.2 μm membrane filter and stored at -70 °C until use.

Cell culture and maintenance. Human normal fibroblast-like cervical (HNCF-PI 52) and cervical cancer (HeLa) cell lines were 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 37 °C in a humidified 5 % CO_2 atmosphere, and were plated 24 h before treatment to allow adherence.

MTT assay and trypan blue staining. Cell growth inhibition was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit (Sigma), as described by Denizot & Lang (1986). A total of 5 x 10^4 to 5 x 10^5 cells from each cell line were seeded in 96-well plates to determine the optimal cell number for MTT assay; 1 x 10^4 HeLa cells and 5 x 10^4 HNCF cells were then seeded in each well containing 100 μl standard medium. After overnight growth, cells were treated for 24 h with 1, 2, 5, 10, 15, 20, 40, 60, 80 and 100 % (v/v) lactobacilli components and live bacteria. Plates were incubated at 37 °C 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

Cell viability was also assessed by the trypan blue dye exclusion method (Sigma).

Lactate dehydrogenase (LDH) assay. LDH activity was assessed using a standardized kinetic determination kit (Sigma). LDH activity was measured in floating dead cells and viable adherent cells. LDH released in the culture supernatant (extracellular LDH or LDHe) was used as an index of necrotic death, while LDH in adherent viable cells was used as an index of intracellular LDH (LDHc). Percentage apoptotic and necrotic cell death was calculated as follows (Peng et al., 2009):
apoptosis (%)=[LDHc/(LDHc+LDHd+LDHe)]×100
 necrosis (%)=[LDHd/(LDHc+LDHd+LDHe)]×100

Caspase-3 activity assay. To investigate whether lactobacilli treatment modulates apoptosis in HeLa and HNCF cells, a colorimetric caspase-3 activity assay was employed (Sigma). Cells were treated with different concentrations of lactobacilli at 37 °C for 24 h in 5 % CO_2. Following trypsinization and centrifugation at 600 g for 5 min, cell pellets were lysed with 50 μl lytic solution by vigorous pipetting and 15 min incubation at 4 °C on an ice bath. Cells were centrifuged at 17 000 g at 4 °C for 15 min, and supernatants containing caspase-3 were stored for further analysis. Protein concentration of supernatants was quantified by measuring the absorbance at 280 nm using the NanoDrop 2000c spectrophotometer (Thermo Scientific). To provide further support for the concentrations obtained, some specimens were investigated by β-actin antibody and Western blotting analyses. After adjusting concentrations, 5 μl each specimen was incubated with 85 μl assay buffer plus 10 μl caspase-3 substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVDD-pNA) in a 96-well plate at 37 °C for 2 h. Absorbance was measured at 405 nm using an ELISA reader. Caspase-3 activity in each culture was expressed as an arithmetical mean ± SD from triplicate measurements. Changes in caspase-3 activity were determined using the formula:
caspase-3 activity (%)=[(sample absorbance−blank absorbance)/control absorbance]×100

Gynaecological tissues. Gynaecological tissue specimens were obtained from patients who underwent surgery at Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran. Ten samples of epithelial carcinoma of the uterine cervix, five of myometrium, five of normal cervix and five placenta (used as controls) were obtained. The study was approved by the Tehran University of Medical Sciences Ethics Review Board and all patients provided informed consent.

RNA isolation, cDNA synthesis and quantitative RT-PCR (qRT-PCR). The FastPure RNA kit (Takara Bio) was used to isolate total RNA from cultured cells and gynaecological tissue specimens. RNA concentration was assessed spectrophotometrically using a Nanodrop 2000c spectrophotometer (Thermo Scientific). Changes in mRNA expression of desired genes were analysed by quantitative PCR (qPCR) after reverse transcription of 1 μg RNA from each sample with the PrimeScript RT reagent kit (Takara Bio). qRT-PCR was performed for mRNA quantification of caspase-3 and HPRT genes on a LightCycler instrument (Roche Diagnostics) using SYBR Premix Ex Taq (Takara Bio). The TaqMan probe used in this study for mRNA quantification of hCGβ gene was described by Giovangrandi et al. (2001). Thermal cycling conditions comprised an initial denaturation at 95 °C for 1 min, and 50 cycles at 95 °C for 15 s and 65 °C for 1 min. The PCR was performed in a final volume of 20 μl containing 10 μl SYBR Green master mix, 2 μl cDNA, 0.5 μl each forward and reverse primer (10 pmol) and 7 μl nuclease-free water. For TaqMan PCR, 0.4 μl each forward and reverse primer (10 pmol), 0.8 μl probe (10 pmol) and 6.8 μl nuclease-free water were used. Experiments were performed in triplicate for each data point. Hypoxanthine phosphoribosyltransferase (HPRT) mRNA was amplified as a normalizer, and fold changes in each target mRNA expression relative to HPRT were calculated. Melting curve analysis was used to validate whether primers yielded a single PCR product.

Statistical analysis. The total expression ratio of the caspase-3 and hCGβ 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), caspase-3 activity, and percentage apoptosis and necrosis of cells treated with various lactobacilli components 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 represented statistical significance.

RESULTS
To assess the lactobacilli component with the highest cytotoxic effect on cervical cells, different components, including supernatants (MRS and RPMI), homogenates,
cytoplasmic extracts, cell-wall extracts and live lactobacilli, were examined. pH- and lactate-adjusted culture media and components were used as controls in some experiments.

**Supernatant of L. gasseri (LGS) and supernatant of L. crispatus (LCS) inhibit HeLa cell proliferation more than HNCF cell proliferation**

Cell growth inhibition was measured by MTT assay. The IC₅₀ value of LGS and LCS against HeLa cells was 5% (v/v) and against HNCF cells was 15% (v/v). Thus, LGS and LCS are less cytotoxic against normal cervical cells, HNCF, than cervical tumour cells, HeLa (P<0.05). The cytotoxic effects of LGS and LCS against HeLa cells are higher than those of MRL (MRS with pH adjusted to that of LGS and LCS) and are concentration dependent. By contrast, the effects of LGS and LCS against HNCF cells are equal to those of MRL at similar concentrations (Fig. 1a, b). These indicate that the higher inhibitory effect of LGS and LCS on HeLa cells than on HNCF cells is lactate and acidity independent. LGSN and LCSN (LGS and LCS with pH adjusted to that of MRS) showed cytotoxic effects similar to those of MRS against HNCF cells, but LGSN and LCSN had higher inhibitory effects on HeLa cells than MRS. These results revealed that the main cause of cervical tumour cell death was not the acidity. It could be the result of a substance other than lactate in the supernatant of the lactobacilli that causes cervical tumour cell death. Meanwhile, the cell line behaviours were similar in trypan blue staining and MTT assay (data not shown).

**Lactobacilli homogenates and cytoplasmic extracts exert the same cytotoxic effects**

Treatment of HeLa cells with increasing concentrations of L. gasseri ATCC 33323 homogenates for 24 h exerted more cytotoxicity than treatment with L. crispatus strain SJ-3C-US homogenates (Fig. 2). There was no significant difference between lactobacilli homogenates and their cytoplasmic extracts for inhibitory effects on either cell line. However, cell-wall extracts did not exert any antiproliferative effect on the cell lines compared to PBS at similar concentrations. Live lactobacilli co-cultured with HeLa cells exerted inhibitory effects at concentrations equal to those of LGS and LCS, but they did not exert any effects on HNCF cells. Notably, lactobacilli-conditioned media supernatants had no significant cytotoxic effects on either cell line (data not shown).

**LGS and LCS decrease the apoptosis to necrosis ratio in HeLa cells**

To further characterize LGS- and LCS-induced cell death, the ratio of LDH released from viable cells, floating dead cells and the culture medium was compared. The percentage apoptotic and necrotic HeLa cell death was 12 and 17 %, respectively (Fig. 3a). After 24 h treatment with LGS, these changed to 9 and 8 %, while after treatment with LCS, they changed to 7 and 3 %, respectively. The lower number of apoptotic cells observed after treatment with LS demonstrates anti-apoptotic effects of LS on HeLa cells.

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**Fig. 1.** Cell growth inhibitory effects of different concentrations of LGS, LCS, LGSN, LCSN, MRL, MRH and MRS on HeLa cells (a) and HNCF cells (b) measured by MTT assay. Each point represents the mean value of three separate experiments.
**LGS and LCS reduced caspase-3 activity**

An *in vitro* colorimetric caspase-3 activity assay was performed to compare apoptosis between control and treated cells. Increasing concentrations of LGS and LCS significantly reduced the activity of caspase-3, a hallmark apoptotic enzyme, compared to MRS- and MRL-treated controls (Fig. 3b). This indicated that the apoptotic inhibition of LS was independent of pH and lactate.

**LGS and LCS upregulate hCGβ mRNA**

hCGβ mRNA levels in gynaecological tissue specimens and HeLa cells were measured by TaqMan qPCR. hCGβ mRNA expression was detected in cervical carcinoma tissues, HeLa cells and placenta, but not in the normal cervix and uterine myometrium. Furthermore, LGS and LCS treatment induced concentration-dependent augmentation of hCGβ expression. After 24 h treatment with 10% (v/v) LGS, LCS and MRS on HeLa cells, LGS and LCS upregulated hCGβ mRNA levels, but MRS alone did not have any significant effect (Fig. 4).

**LGS and LCS downregulate caspase-3 mRNA levels**

To investigate whether treatment with LS triggered apoptosis through the transcriptional activity of caspase-3, we evaluated the effects of LGS, LCS and MRS on caspase-3 mRNA levels. Fig. 4 shows that treatment with LS reduced transcriptional levels of caspase-3, which was consistent with LDH and caspase-3 colorimetric assay results.

**DISCUSSION**

Cervical cancer is an HPV-related cancer occurring in only a small proportion of individuals exposed to this very common infection. Most HPV infections and a significant proportion of HPV infections are asymptomatic.
Data are expressed as the mean ± SEM of three experiments. Asterisks indicate *P<0.01.

Fig. 4. HeLa cells were treated with 10% (v/v) LGS, LCS and MRS to investigate whether exposure to lactobacilli components affected changes in caspase-3 and hCGβ gene expression levels. Dark grey bars show fold changes in caspase-3 mRNA expression quantified by qPCR and light grey bars indicate hCGβ mRNA expression assessed by TaqMan PCR (compared to the control). Data are expressed as the mean ± SEM of three experiments.

proportion of HPV-induced neoplastic lesions resolve spontaneously (Boccardo et al., 2010), and hence, other factors must be involved in invasive cervical carcinoma development after HPV infection. One suspected factor is the cervical environment and its microbial flora. The normal cervical and vaginal microbial flora are dominated by lactobacilli, which are known probiotics. Because of the reported antitumour effects of probiotics, their inhibitory effects on BV, and the relationship between BV and CIN, we hypothesized a correlation between vaginal lactobacilli and cervical cancer.

Probiotic bacteria have antitumour activities (Russo et al., 2007), but their precise mechanism of cell growth inhibition is only partially elucidated. Probiotic effects are caused by different components. HGC-27 and DLD-1 cells were resistant to L. rhamnosus GG cell-wall fractions, whereas increasing cytoplasm fraction concentrations induced an evident antiproliferative effect (Orlando et al., 2009). Cell-bound exopolysaccharide from Lactobacillus acidophilus is antitumorigenic against colon cancer cells (Kim et al., 2010). Peptidoglycans isolated from Bifidobacterium infantis strain ATCC 15697 have antitumour activity (Sekine et al., 1995). Thus, we compared the antiproliferative effects of the supernatants, cytoplasmic extracts, cell-wall extracts and live lactobacilli on normal and tumour cervical cell lines, and demonstrated that the most potent cytotoxic effects caused by lactobacilli are because of their culture supernatants.

Some probiotic activities result from the lactate production and pH of cultures (Lan et al., 2007). We compared the effects of the LS with those of MRL and MRH. Interestingly, MRL showed a more potent inhibiting effect on both cell lines than MRH despite having similar pH. Therefore, in the present study, there is a difference between lactate production and pH since lactate production was beyond the acidic pH range. Also, lactate production was a more important part of the lactobacilli inhibitory effect than pH alone.

Regarding cancer cell-selective effects, HK cells of L. acidophilus 606 inhibit cancer cell proliferation but are much less cytotoxic to normal cells (Choi et al., 2006). Considering that vaginal lactobacilli colonize the cervix of healthy adults, we compared the effects of these lactobacilli between normal and tumour cervical cell lines. Interestingly, live lactobacilli co-cultured with a normal cervical cell line (HNCF) did not show any cytotoxic effect after 24 h, but were potent growth inhibitors of cervical tumour cells (HeLa). On comparing the effects of LS on these cell lines, it was concluded that although both supernatants had cytotoxic effects, they were much less effective against normal cells. Notably, their effects were comparable with those of MRL as HNCF cells responded to supernatants in a manner similar to their response to MRL, but HeLa cell responses to supernatants and MRL were clearly different. Since a limitation of many cancer therapeutics is their toxic effects on normal cells and tissues (Damia & Broggini, 2004), we can hypothesize that there are non-lactate molecules in LS that have antitumour activities but these are safe for normal tissues.

Several mechanisms have been proposed to account for the antitumour effects of probiotics. Soluble polysaccharides from L. acidophilus 606 resulted in the death of HT-29 cancer cells by inducing apoptosis (Choi et al., 2006). However, probiotic bacteria also exert their effects by inhibiting apoptosis. For example, probiotic cell lysate administration might reduce mitochondria-mediated oxidative stress and subsequent apoptosis in acetaminophen-induced hepatotoxicity (Sharma et al., 2011). Bifidobacterium bifidum reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis (Khailova et al., 2010). We demonstrated that LS exert an anti-apoptotic effect on HeLa cells because they reduce caspase-3 expression and activity. Reduction of LDH released by floating dead cells indicates a lower ratio of apoptotic cells in LS-treated cells. It should be noted that this anti-apoptotic effect was clearly lactate independent.

Although apoptosis is a cell death pathway, a complex balance between proliferation and apoptosis maintains normal cell turnover and any imbalance leads to tumour formation. Caspase-3 expression is higher among patients with CIN3 than among those with CIN1, although CIN3 has more invasive potential than CIN1 (Cheung et al., 2002). Further, some malignant cell types respond to antitumour agents (Del Canto et al., 2007) and cell-bound exopolysaccharides from probiotic bacteria (Kim et al., 2010) that induce autophagic cell death. Our data suggest that the anti-apoptotic effect of lactobacilli may result from hCGβ expression upregulation and autophagy, as many studies have reported that hCGβ expression restrains...
apoptosis but not autophagy (Del Canto et al., 2007; Jankowska et al., 2008b).

In conclusion, our results show for what is believed to be the first time that normal and tumour cervical cells respond differently to vaginal lactobacilli, independent of pH and lactate, and confirm that elevated hCGβ expression is consistent with apoptotic inhibition. Our results also encourage further studies on the interaction between lactobacilli and cervical cells, and administration of common vaginal lactobacilli as probiotics for cervical cancer prevention.

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