Cytotoxic effects of various lactic acid bacteria on Caco-2 cells

Sevda ER[1],*, Ayşe Tansu KOPARAL[2], Merih KIVANÇ[2]

[1]Department of Microbiology, School of Pharmacy, İstanbul Medipol University, İstanbul, Turkey
[2]Department of Biology, Faculty of Sciences, Anadolu University, Eskişehir, Turkey

Abstract: Probiotics are live microbial food supplements that can be considered a functional food. They benefit the health of a host animal by maintaining their intestinal microbial balance. Most probiotic microorganisms are lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. LAB have been reported to possess certain anticancer properties. The vast majority of studies on their anticancer effects have dealt with colorectal cancers, although there have also been some studies on breast and bladder cancers. Colon cancer is the fourth most common cause of cancer-related mortality in the world. The aim of this study was to investigate the antiproliferative effects of the cell-free filtrate and the cell-free lyophilized filtrate of 3 LAB (*Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *Weissella confusa*) on human colorectal adenocarcinoma cell line Caco-2. The filtrates were found to inhibit the growth of colon cancer cells in a dose-dependent manner as detected by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, suggesting that these strains might have use as probiotics in functional food or for colon cancer treatment. There are no other studies related to the anticancer activities of *W. confusa* in the literature.

Key words: Probiotics, lactic acid bacteria, cell-free filtrate, Caco-2, MTT assay

1. Introduction

Probiotics are live microbial food supplements that can be considered a functional food. Probiotics have been the focus of intense research in recent years. They benefit the health of a host animal by maintaining their intestinal microbial balance (Fuller, 1991).

The gut surface, which constitutes the first physical barrier to intestinal and orally ingested bacteria, is formed by a single layer of intestinal epithelial cells (IEC). The bacteria mentioned include both probiotics and those that are naturally present in fermented food products (Abreu, 2010). Lactic acid bacteria (LAB) are often used as supplements in probiotic products. The therapeutic effects of LAB include improvement of lactose intolerance, prevention of intestinal infection, decrease in serum cholesterol, increase in immune response, anticarcinogenic activity, and antioxidative effects (Lin and Chang, 2000).

LAB have been reported to possess certain anticancer properties (Kim et al., 2002a). LAB have antitumor properties that inactivate or inhibit carcinogenic compounds in the gastrointestinal tract, stimulate the immune response, and reduce the enzymatic activity of b-glucuronidase, azoreductase, and nitroreductase, which are known to convert precarcinogens into

carcinogens (Vamanu et al., 2006). Moreover, LAB have been shown to increase colonic NADPH-cytochrome P-450 reductase activity (Pool-Zobel et al., 1996) and glutathione S-transferase levels (Challa et al., 1997). Additionally, they have been shown to reduce hepatic uridine diphosphoglucuronyl transferase activity, which is involved in the metabolism of carcinogens in rats (Abdelali et al., 1995).

The vast majority of studies on these anticancer effects deal with colorectal cancer (Rafter, 2003). Colon cancer is a serious health problem in the majority of developed countries; it remains the leading cause of cancer mortality throughout the world (Pisani et al., 1993). The precise mechanisms by which LAB inhibit colon cancer are unknown. Several mechanisms have been suggested, such as elevation of the host's immune response, binding and degradation of potential carcinogens, qualitative alterations in the intestinal microflora that produce putative carcinogens and promoters (e.g., bile–acid-degrading bacteria), production of antitumorigenic or antimutagenic compounds in the colon, and alteration of the metabolic activities of intestinal microflora (Hirayama and Rafter, 2000; Kim et al., 2008).

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* Correspondence: ser@medipol.edu.tr

Epidemiological studies have shown that diet plays a role in the etiology of most large intestine cancers and many studies have confirmed the effect of endogenous microflora on the onset of colon cancer. Altering the intestinal microflora may affect tumor development and thus considerable attention has been focused on dietary supplements that can affect the gut microflora as a strategy for colorectal cancer prevention (Rafter, 2002). Furthermore, mechanistic studies have suggested that probiotic bacteria or their byproducts influence epithelial cell kinetics in the colon, decreasing cancer cell proliferation (Sanders, 1999).

In the present study, we aimed to evaluate the antiproliferative properties of the cell-free filtrate and the cell-free lyophilized filtrate of 3 LAB (*Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *Weissella confusa*) by using an MTT assay on a human cell line, namely the human epithelial colorectal adenocarcinoma Caco-2 cell line as an intestinal model in light of this tissue's direct contact with food. The cell-free filtrate and the cell-free lyophilized filtrate of the 3 tested LAB were found to inhibit the growth of Caco-2 cells in a dose-dependent manner as detected by the MTT assay. This suggested that these strains could be used as potential probiotics in dairy foods for the prevention of colon cancer. Additionally, there are no studies related to the anticancer activity of *W. confusa* in the literature.

2. Materials and methods

2.1. Bacterial culture and supernatant
Bacteria that had been isolated from meat and identified in an earlier study were used (Dinçer and Kıvanç, 2012) (*P. pentosaceus* 48.P3.3, *L. plantarum 154*.P7.2, and *W. confusa* 163.P5.8). The strains were grown in de Man, Rogosa, and Sharpe medium at 37 °C for 24–48 h anaerobically. For the in vitro preparation of the cell-free filtrate, cultured bacterial cells were centrifuged at $11,000 \times g$ for 30 min. The supernatants were filtered using a 0.22-µm syringe filter. The pH of the supernatants was adjusted to 7 with NaOH and stored at +4 °C until use.

2.2. Caco-2 cell culture
The cell line used in this study was human colorectal adenocarcinoma Caco-2. The cell line was obtained from the ŞAP Institute of Turkey (Ankara). The Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) F-12, including 10% fetal bovine serum (FBS), 1% (v/v) L-glutamine, 1% (v/v) penicillin-streptomycin solution, and 7.5% $NaHCO_3$. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and subcultivated at 70% to 80% confluence.

2.3. Cell viability test (MTT assay)
Cell viability was determined using the MTT assay. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt converted to an insoluble purple formazan by the cleavage of the tetrazolium ring through the succinate dehydrogenase within the mitochondria. Since the cell membranes are impermeable to the formazan product, the product accumulates in healthy cells (Mossman, 1983). Briefly, 15×10^3 Caco-2 cells/well were incubated in 96-well plates and cultured for 24 h. The culture medium was removed and different concentrations of cell-free filtrate and cell-free lyophilized filtrate were added to the culture medium. Noninoculated DMEM F-12 medium was used as a negative control. The Caco-2 cells were further incubated for 8 h and 24 h. After incubation, cell viability was determined using the colorimetric MTT assay. MTT solution (100 µL/mL) was added to each well. After 2 h of incubation at 37 °C, 100 µL of dimethylsulfoxide (DMSO) was added to dissolve the blue crystals and absorbance was read. The optical density was measured at 570 nm using a microplate reader (Bio-Tek, ELX808IU, USA). The assay was performed in triplicate. SPSS was used for the statistical analyses of the MTT assay results. The data were evaluated using one-way analysis of variance followed by the Tukey test. A value of $P < 0.05$ was considered significant.

3. Results
Dose-dependent cytotoxic effects of the cell-free filtrates and cell-free lyophilized filtrates of *P. pentosaceus*, *L. plantarum*, and *W. confusa* on Caco-2 cell proliferation were observed.

3.1. Cytotoxic effects of *Pediococcus pentosaceus* on Caco-2 cells
Figure 1 shows the proliferation of Caco-2 cells in the presence of the cell-free filtrate (Figure 1a) and the cell-free lyophilized filtrate (Figure 1b) of *Pediococcus pentosaceus*. At concentrations of 0.005 µL/mL, 0.05 µL/mL, 0.5 µL/mL, 5 µL/mL, and 50 µL/mL of *P. pentosaceus* cell-free filtrate, a weak inhibition of cellular proliferation (2%–8%) was observed at 8 h and 24 h of incubation. The cytotoxicity inhibition rates were 9% and 25% for a concentration of 500 µL/mL at 8 h and 24 h of incubation, respectively.

At concentrations of 0.1 µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL, and 1000 µg/mL of cell-free lyophilized *P. pentosaceus* filtrate, a weak inhibition of cellular proliferation (5%–15%) was observed at 8 h and 24 h of incubation. The cytotoxicity inhibition rates were 21% and 33% at a concentration of 10,000 µg/mL at 8 h and 24 h of incubation, respectively.

According to these results, the strongest effect of the *P. pentosaceus* filtrate was found at a concentration of 10,000 µg/mL of the cell-free lyophilized filtrate at 24 h of incubation (33% inhibition).
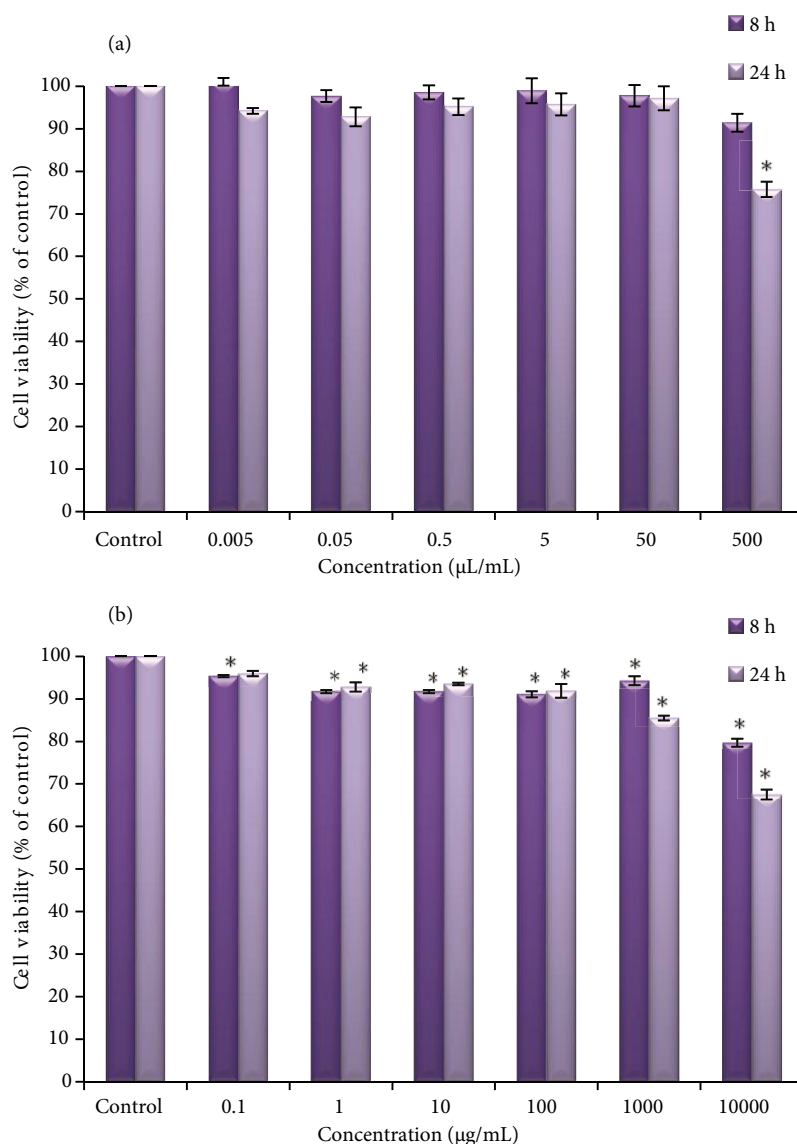
Figure 1. Proliferation of Caco-2 cells in the presence of *Pediococcus pentosaceus*. Cell proliferation was determined using the MTT colorimetric assay. Cell proliferation was measured at an optical density of 570 nm. * = significant effect (P < 0.05) for (a) the cell-free filtrate of *Pediococcus pentosaceus* and (b) the cell-free lyophilized filtrate of *Pediococcus pentosaceus*.

3.2. Cytotoxic effects of *Lactobacillus plantarum* on Caco-2 cells

Inhibition of Caco-2 cell proliferation by the cell-free filtrate (Figure 2a) and the cell-free lyophilized filtrate (Figure 2b) from *Lactobacillus plantarum* is shown in Figure 2. At concentrations of 0.005 µL/mL, 0.05 µL/mL, 0.5 µL/mL, and 5 µL/mL of *L. plantarum* cell-free filtrate, a weak inhibition of cellular proliferation (5%–18%) was observed at 8 h and 24 h of incubation. Nevertheless, the cytotoxicity inhibition rates were 26% and 50% for the 500 µL/mL filtrate at 8 h and 24 h of incubation, respectively.

At concentrations of 0.1 µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL, and 1.000 µg/mL of *L. plantarum* cell-free lyophilized filtrate, a weak inhibition of cellular proliferation (0%–14%) was observed at 8 h and 24 h of incubation. The cytotoxicity inhibition rates were 33% for a concentration of 10,000 µg/mL at 8 h and 24 h of incubation.

According to these results, the strongest effect of *L. plantarum* was found at a concentration of 500 µL/mL of the cell-free filtrate at 24 h of incubation (50% inhibition).
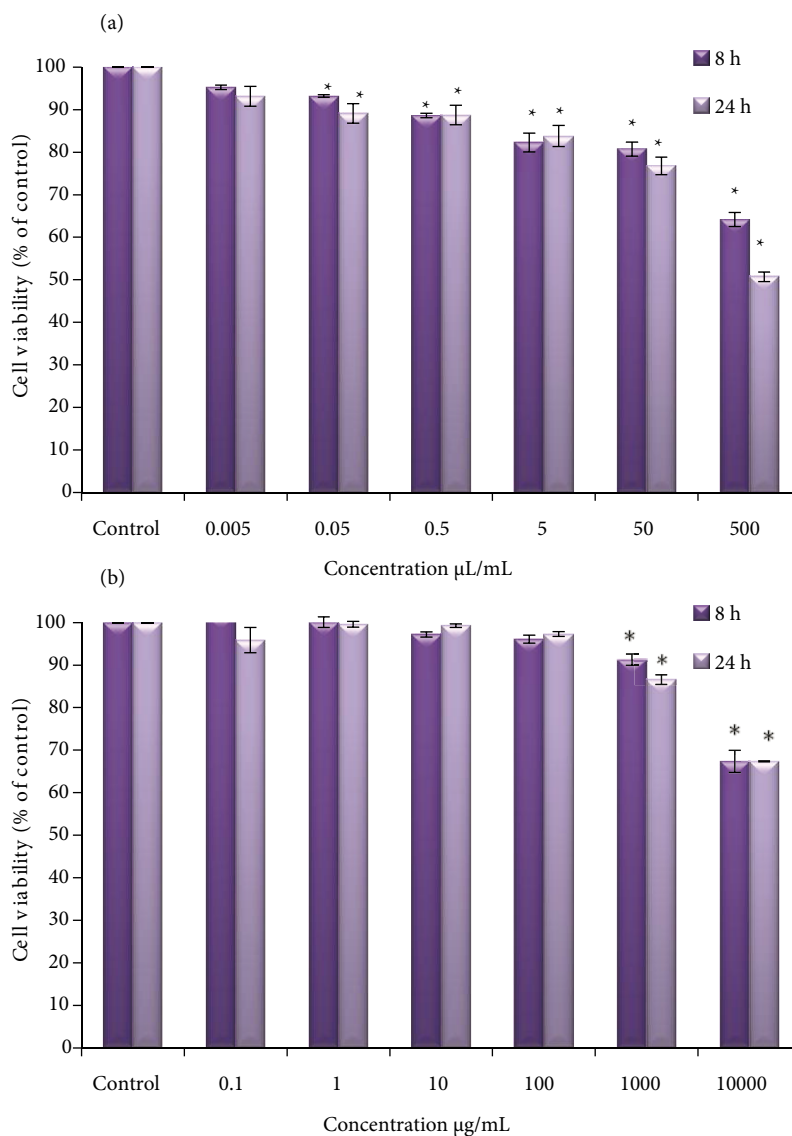
(a)

(b)

Figure 2. Proliferation of Caco-2 cells in the presence of *Lactobacillus plantarum*. Cell proliferation was determined using the MTT colorimetric assay. Cell proliferation was measured at an optical density of 570 nm. * = significant effect (P < 0.05) for (a) the cell-free filtrate of *Lactobacillus plantarum* and (b) the cell-free lyophilized filtrate of *Lactobacillus plantarum*.

3.3. Cytotoxic effects of *Weissella confusa* on Caco-2 cells
Figure 3 shows proliferation of Caco-2 cells in the presence of the cell-free filtrate (Figure 3a) and the cell-free lyophilized filtrate (Figure 3b) of *Weissella confusa*. At concentrations of 0.005 μL/mL, 0.05 μL/mL, 0.5 μL/mL, and 5 μL/mL of *W. confusa* cell-free filtrate, a weak inhibition of cellular proliferation (0%–7%) was observed at 8 h and 24 h of incubation. The cytotoxicity inhibition rate was 20% for a concentration of 50 μL/mL at 24 h of incubation. The cytotoxicity inhibition rates were 11% and 37% for a concentration of 500 μL/mL at 8 h and 24 h of incubation, respectively.

At bacterial concentrations of 0.1 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL, and 1000 μg/mL of the *W. confusa* cell-free lyophilized filtrate, a weak inhibition of cellular proliferation (0%–14% of inhibition) was observed at 8 h and 24 h of incubation. The cytotoxicity inhibition rates were 9% and 32% for a concentration of 10,000 μg/mL at 8 h and 24 h of incubation, respectively.

According to these results, the strongest effect of *W. confusa* was found at a concentration of 500 μL/mL of the cell-free filtrate at 24 h of incubation (37% inhibition).
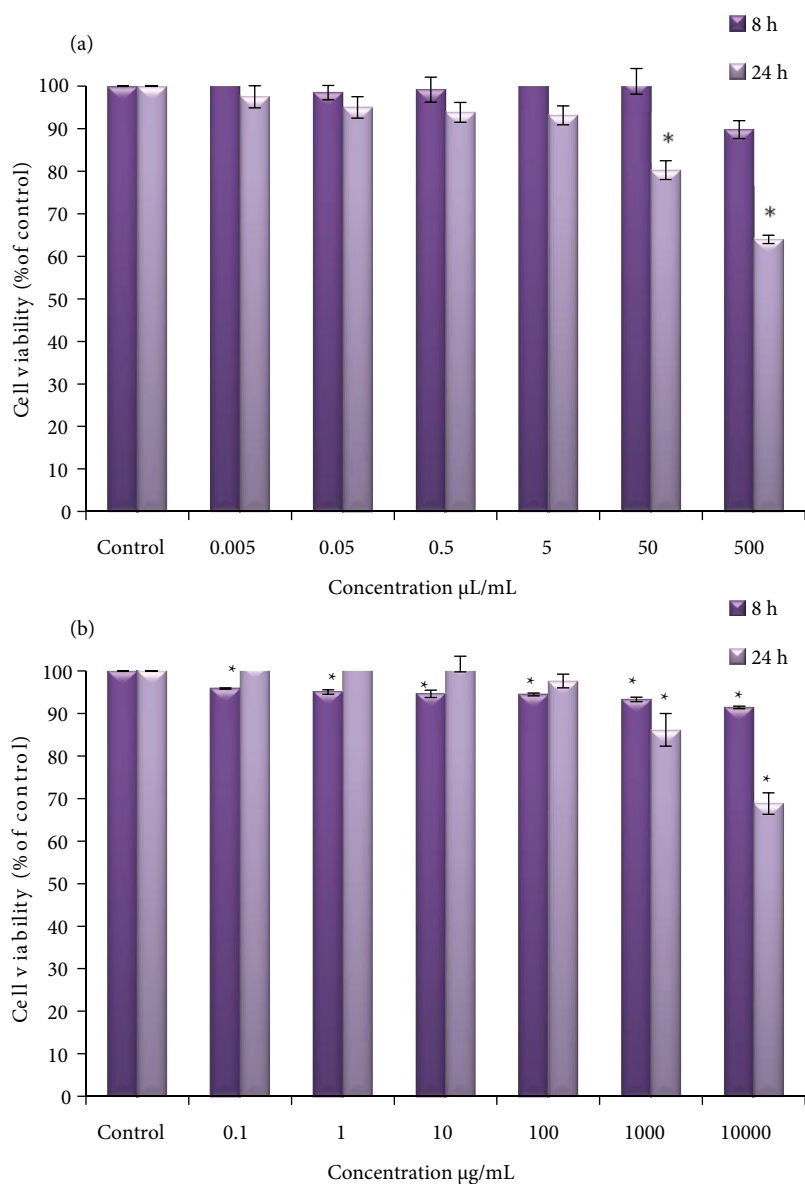
Figure 3. Proliferation of Caco-2 cells in the presence of *Weissella confusa*. Cell proliferation was determined using the MTT colorimetric assay. Cell proliferation was measured at an optical density of 570 nm. * = significant effect (P < 0.05) for (a) the cell-free filtrate of *Weissella confusa* and (b) the cell-free lyophilized filtrate of *Weissella confusa*.

4. Discussion

Probiotics are live microorganisms that, when administered in adequate amounts, provide a health benefit for the host (FAO/WHO, 2001). Generally, probiotics affect the host beneficially by improving intestinal microbial balance, improving immune function, and protecting against colon cancer (Wang et al., 2014). The IEC monolayers are proven to be adequate models to test the bacterial adhesion to intestinal epithelium, which constitutes one criterion that must be met for the selection of potential probiotics

(Monteagudo-Mera et al., 2010; Lopez et al., 2012). In addition, there are several references in the literature telling us that probiotic bacteria, especially *Lactobacillus* and *Bifidobacterium*, elicit a strain-specific cytokine pattern through their ability to interact with the IEC (Morita et al., 2002; Bahrami et al., 2010; Boesten et al., 2011).

The inhibitory effects of probiotics upon the proliferation of several colon cancer cell lines have been previously demonstrated (Choi et al., 2006). Ewaschuk et al. (2006) reported that *Lactobacillus acidophilus*, *L.*

bulgaricus, *L. casei*, *L. plantarum*, *Bifidobacterium breve*, *B. infantis*, *B. longum*, and *Streptococcus thermophilus* reduced the viability and induced apoptosis of HT-29 and Caco-2 cells (Ewaschuk et al., 2006). The Caco-2 (colon adenocarcinoma) cell line is one of the most used in vitro models for the study of intestinal absorption of compounds at the screening level. These cells are seeded on semi-permeable membranes, on which they form a continuous monolayer with tight junctions, mimicking the intestinal barrier. Thus, they are able to differentiate and polarize in a long-term culture. Although the complexity of the in vivo processes may cast some doubts on the full representativeness of the model, it has become a popular surrogate for human intestinal epithelium (Turco et al., 2011). For this reason, we used Caco-2 cells in our study.

Lactobacilli and bifidobacteria are the most prominent probiotic bacteria, and have been accepted as the reason for the increasing research attention on the prevention of cancer (Wang et al., 2014). The different fractions of LAB, such as whole cells, heat-killed cells, the cell wall, peptidoglycan, and cytoplasmic fraction, all have preventive effects against human cancer cell lines (Kim et al., 2003). Anticancer activities were found in peptidoglycans isolated from *Lactobacillus casei* (Fichera and Giese, 1994). Furthermore, it has been reported that polysaccharide fractions originating from *Lactobacillus* cultures (Oda et al., 1983) and glycoproteins found in the supernatants of *Lactobacillus* cultures (Manjunath and Ranganathan, 1989) have the same effect. In our study, we evaluated the antiproliferative effects of the cell-free filtrate and the cell-free lyophilized filtrate of 3 LAB (*P. pentosaceus*, *L. plantarum*, and *W. confusa*) on the human colorectal adenocarcinoma cell line Caco-2. The cell-free filtrate and the cell-free lyophilized filtrate of the 3 LAB were found to inhibit the growth of colon cancer cells in a dose-dependent manner as detected by the MTT assay. In vitro toxicology and related sciences have traditionally evaluated the effects of various agents on cell growth and proliferation by observing the changes in cell numbers and in cell morphology. Current standard approaches include the assays conducted to measure distinct cell growth and metabolism related endpoints, such as the activity of intracellular enzymes, integrity of cellular membranes, DNA synthesis, and ATP status (Schröterova et al., 2009). Among the most often used assays are those that are conducted to measure the metabolic activity of viable cells using colorimetric changes based on tetrazolium salt reduction. The MTT assay utilizes 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to blue colored formazan by metabolically active cells. The intensity of the color of the dissolved formazan is proportional to the number of viable cells. The MTT assay was tested for its validity with various cell lines (Mossmann, 1983).

It is clear that cell culture-based toxicity tests are of interest, due to having the potential to screen samples for a biochemical response while retaining the ability to detect more general cytotoxicity endpoints (Puerto et al., 2009). Paolillo et al. (2009) studied the cytotoxic effect of live cells of *L. plantarum* on Caco-2 cells. In this study, 1 × 10⁶ Caco-2 cells/well were used on a 96-well plate. The effect of *L. plantarum* on the viability of cultured Caco-2 cells was examined by MTT assay. Although *L. plantarum* exhibited no significant effect on the viability of Caco-2 cells 12 h posttreatment compared to cells alone, after 24 h and 48 h of exposure, the number of viable cells was 92 ± 3% and 91 ± 3% respectively, both higher than the controls (73.6 ± 2.9% and 68.25 ± 2.8%, respectively) (Paolillo et al., 2009). In our study, cell proliferation at 24 h of incubation was often higher than cell proliferation at 8h of incubation. According to these results, we can conclude that incubation time may affect antiproliferative activity.

A dose-dependent response has been reported for the anticarcinogenic and/or antimutagenic abilities of some LAB strains (Salminen et al., 1998). Zabala et al. (2001) evaluated whether or not suspensions of the bacteria *Enterococcus faecium* CH3 and *Lactobacillus salivarius* HA8 have an effect on proliferation of myeloma cells. At bacterial concentrations of 10⁶–10⁷ cfu/mL, a weak inhibition of cellular proliferation (70.9 ± 81.5% of survival) was observed for both strains. However, they displayed a strong inhibitory effect (16.7% and 5% for *E. faecium* HN1 and *L. salivarius* HA8, respectively) at a concentration of 10⁸ cfu/mL (Zabala et al., 2001). We also observed a dose-dependent response for the antiproliferative abilities of *P. pentosaceus*, *L. plantarum*, and *W. confusa* in our study. With respect to the proliferation of Caco-2 cells, strong inhibition was found with the cell-free filtrate of *L. plantarum* (500 µL/mL) after 24 h of incubation. Some strains are better candidates as probiotics, but no strain has all the features of a probiotic (Cebeci and Gürakan, 2003). According to our results, the cell-free filtrate of *L. plantarum* has stronger effects compared to those of *P. pentosaceus* and *W. confusa*.

In conclusion, this work has shown that *P. pentosaceus*, *L. plantarum*, and *W. confusa* have the potential to inhibit the proliferation of Caco-2 cells. *L. plantarum* showed the strongest inhibitory effect. Although there are anticancer studies with *L. plantarum* (Kim et al., 2002b; Paolillo et al., 2009), there are not adequate data regarding the anticancer effects of *P. pentosaceus* and *W. confusa*. By using the MTT assay, Patel et al. (2010) reported that dextran isolated from *P. pentosaceus* has no cytotoxic effect on HeLa cells (human cervical cancer). The dextran isolated from *P. pentosaceus* possesses potential as a gelling agent in food formulations and as a drug delivery carrier, tissue-

engineering scaffold, and as a biomaterial for various other biomedical applications (Patel et al., 2010). Additionally, Villarante et al. (2011) revealed that bacteriocin isolated from *Pediococcus acidilactici* has a cytotoxic affect on HT29 (human colon adenocarcinoma) and HeLa cells as detected by MTT assay. However, there are no studies related to the anticancer activity of *W. confusa* in the literature. This study suggests that *P. pentosaceus* and *W. confusa* may also be candidates for probiotic use. More work is needed in order to reveal the causative underlying characteristics responsible for specific antitumor effects.

In the modern era, there is a demand for such foods as functional foods, pharma foods, and nutraceuticals for preventing diseases (Khan, 2014). Increasing interest has stimulated innovation and new product development in the food industry around the world (Vinderola, 2008).

For example, probiotic products including LAB are widely developed. There are preparations of *L. plantarum* in markets (Bioculture, Quest) but not preparations of *P. pentosaceus* or *W. confusa*. Hence, products including these bacteria may be developed as an alternative to the products that are already on the market. This study suggests that *P. pentosaceus* and *W. confusa* may also be candidates for probiotic use. More work is needed in order to reveal the causative underlying characteristics responsible for specific antitumor effects.

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