Antiproliferation effects and antioxidant activity of two new *Lactobacillus* strains

Efeitos de antiproliferação e atividade antioxidante de duas cepas novas de Lactobacillus

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■ Abstract

The microorganisms most commonly used as probiotics are lactic acid bacteria, especially those of the genus *Lactobacillus*. In the present study, two *Lactobacillus* strains, *L. pentosus* ITA23 and *L. acidipiscis* ITA44, previously isolated from mulberry silage, were characterized for their antiproliferative and antioxidant activities. The antiproliferative effects of the strains were investigated using the MTT assay with breast cancer (MDA-MB-231), liver cancer (HepG2) and normal liver (Chang) cell lines. The strains were tested for their antioxidant activity using the FRAP and ABTS methods. The results showed that the two *Lactobacillus* strains had good antiproliferative effects against both cancer cell lines tested, while their effects on the normal cells were weak. Based on the results of the antioxidant tests, the intact cells and cell-free extracts of the two *Lactobacillus* strains showed more than 135 and less than 50 µg trolox/ml of antioxidant activity, respectively. *Lactobacillus pentosus* ITA23 and *L. acidipiscis* ITA44 can be considered as potential probiotic candidates for humans because of their antioxidant activity and antiproliferation effects against cancer cells.

Keywords: *Probiotic; Lactobacillus; Antiproliferation; Antioxidant.*

■ Resumo

Os micro-organismos mais comumente usados como probióticos são as bactérias lácticas, especialmente aquelas do genus *Lactobacillus*. No presente estudo, duas cepas de *Lactobacillus*, *L. pentosus* ITA23 e *L. acidipiscis* ITA44, previamente isoladas da silagem de amora, foram caracterizados por suas atividades antiproliferativas e antioxidantes. Os efeitos antiproliferativos das cepas foram investigados usando-se o ensaio MTT, com linhas de células cancerígenas de mama (MDA-MB-231) e do fígado (HepG2), e com células normais do fígado (Chang). As atividades antioxidantes das cepas foram testadas utilizando-se os métodos de FRAP e ABTS. Os resultados demonstraram que as duas cepas de *Lactobacillus* tinham bons efeitos antiproliferativos contra as duas linhas de células cancerígenas, enquanto seus efeitos nas células normais eram fracos. Baseando-se nos resultados dos testes antioxidantes, as células intactas e os extratos livres de células das duas cepas de *Lactobacillus* demonstraram mais que 135 e menos que 50 µg trolox/mL de atividade antioxidante, respectivamente. *Lactobacillus pentosus* ITA23 e *Lactobacillus acidipiscis* ITA44 podem ser considerados como potenciais candidatos probióticos para humanos, devido à sua atividade antioxidante e aos efeitos antiproliferativos contra células cancerígenas.

Palavras-chave: *Probiótico; Lactobacillus; Antiproliferação; Antioxidante.*

1 Introduction

Probiotic is defined as "[...] live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [...]" (FAO, 2001, p. 50). Lactic acid bacteria, including the genus *Lactobacillus*, are the most commonly used probiotics in the food industry because of their beneficial functional effects and their reputation as "generally recognized as safe" (GRAS) and environmentally friendly bacteria (SAARELA et al., 2000; SALMINEN et al., 1998; KLAENHAMMER; KULLEN, 1999). Probiotics have become highly recognized as supplements for human consumption because of their beneficial effects on the health and well-being (OOI; LIONG, 2010). For example, interference with pathogens and the prevention of infections, immune system stimulation and immunomodulation, anti-carcinogenic and antioxidant activities, reduction of gastrointestinal disorders such as diarrhoea, constipation and the irritable bowel syndrome, alleviation of allergic and lactose intolerance symptoms, reduction in serum cholesterol, blood pressure and risk of gestational diabetes, and the production of beneficial compounds, such as vitamins, short-chain fatty acids (SCFAs) and conjugated linoleic acid are some of their important beneficial effects (LOLLO et al., 2013, 2015*;* SHOKRYAZDAN et al., 2017). Among these effects, the antiproliferative or cytotoxic effect of probiotic strains against cancer cells is a very important and relatively recent aspect. This probiotic property is important because cancer is considered as the major worldwide cause of morbidity (WHO, 2009). Another recently discovered important functional property of a probiotic strain is its antioxidant activity. Many reactive oxygen species (ROS) are continuously formed in food systems and in the human body (AHOTUPA et al., 1996), and the antioxidant activity of probiotic strains could be a helpful option to encounter the adverse effects of ROS. Therefore, probiotic strains with strong antiproliferative effects and antioxidant activity can be considered as important agents in the food industry for producing functional foods.

Although many different probiotics have already entered the food market, novel strains with more functional probiotic properties than the existing ones are the object of new research due to the growing demand for "healthy" foods in the food industry. In the present study, two previously isolated and identified *Lactobacillus* strains from the author's laboratory were used to investigate their antioxidant activity, and also their antiproliferation effects against two different carcinoma cell lines and one normal cell line as the control, to investigate the selective killing effects of the two *Lactobacillus* strains.

2 Materials and methods

2.1 *Lactobacillus* strains and sample preparation

Two *Lactobacillus* strains, namely *L. pentosus* ITA23 and *L. acidipiscis* ITA44, previously isolated and identified (SHOKRYAZDAN et al., 2015) from locally prepared mulberry (*Morus alba*) silage, were used as test strains. De Man-Rogosa-Sharpe (MRS) broth (Merck, Germany) was used as the growth medium for the *Lactobacillus* strains. The strains were grown at 37 °C in anaerobic jars (Oxoid, UK) containing gaspack (AnaeroGen, Oxoid, UK). Each *Lactobacillus* strain was sub-cultured three times before the experiment, and the overnight culture (final concentration of 1×10^7 CFU/ml) of each strain was centrifuged at $4000 \times g$ for 5 min at 4 °C. The supernatants were collected and filter sterilized through a 0.22 μm pore-size filter (Pall, USA). Filter sterilized supernatants were used as treatments in the antiproliferation assay. The cell pellets were washed three times with deionized water, and freeze dried for use in the antioxidant study. Intact cells [10 mg of each freeze-dried cell sample, re-suspended in 1 ml of PBS (pH 7.2)] and intracellular cell-free extracts were used for the investigation of the antioxidant activity of the two *Lactobacillus* strains. To prepare the intracellular cell-free extract, 10 mg of each freeze-dried cell sample was re-suspended in 1 ml of deionized water, sonicated on ice for 5 min, centrifuged at $7000 \times g$ for 10 min at 4 °C, and the supernatants collected.

2.2 Antiproliferation assay

2.2.1 Cell culture

Breast cancer (MDA-MB-231), liver cancer (HepG2) and normal liver (Chang) cell lines were obtained from the American Type Culture Collection (ATCC). The cell lines were routinely grown to 80% confluent in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% (v/v) foetal bovine serum (FBS, inactivated at 56 °C for 30 min) and 1% (v/v) penicillin–streptomycin (10,000 IU/ml and 10,000 μg/ml). The cells were incubated in a CO_2 incubator at 37 °C in 5% CO_2 for maintenance and during the experiment.

2.2.2 MTT assay

For the MTT assay, filter sterilized supernatants from overnight cultures of each *Lactobacillus* strain were used as the treatment. All the chemicals used in the experiment were from Sigma, USA. The MTT assay followed the method of Haza et al. (2004) with some modifications. Each cell line was placed in a 100 μl/well in 96 well plates (1×10^6 cells/plate). After incubation at 37 °C for 24 h, the cell medium was replaced by a serial dilution of the treatment in fresh RPMI medium at final concentrations of 0, 5, 10, 15, 20, 25, 30 and 35% for MDA-MB-231 and HepG2, and 0, 10, 20, 30, 40, 50, 60 and 70% for Chang

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cells. The plates were then incubated for 24, 48 and 72 h. After incubation, 20 µl of MTT (0.5 mg/ml in phosphate buffer saline) was added to each well and the plates incubated for 3 h in the dark. The mixture was then discarded from the wells and 100 µl DMSO (dimethyl sulfoxide) added to the monolayers of the cells in order to solubilize the formazan precipitates. After 10 min of incubation at room temperature, the absorbance was measured at 570 nm using a micro-plate reader.

The percentage of cell viability was calculated using Equation 1:

$$\text{Cell viability}(\%) = (A_{570}\text{sample} - A_{570}\text{blank}) \times 100 / A_{570}\text{control} \quad (1)$$

Where, A_{570}, absorbance at 570 nm; sample, monolayer of each cell line plus different concentrations of treatment in RPMI medium; blank, different concentrations of treatment in RPMI medium; control, monolayer of each cell line plus RPMI medium.

2.2.3 Antioxidant assay

2.2.3.1 FRAP

The ferric reducing ability of plasma "FRAP" assay devised by Benzie and Strain (1996) with some minor modifications was used to investigate the total antioxidant activity of the *Lactobacillus* strains. An amount of 200 µl of FRAP reagent [300 mM acetate buffer (pH 3.6, containing 6.4 ml 2 M sodium acetate and 93.6 ml 2 M acetic acid), 10 mM 2,4,6-tri (2-pyridyl)-1,3,5-triazine (TPTZ) in 40 mM HCl, and 20 mM ferric chloride in a ratio of 10:1:1 (v:v:v)] was added to 50 µl of intact cells or intracellular cell-free extract of each *Lactobacillus* strain. After incubation of the mixture for 30 min at room temperature in the dark, the absorbance was measured at 593 nm using a spectrophotometer (Barnstead International, USA).

2.2.3.2 ABTS

The ABTS radical cation decolourization assay described by Tsai et al. (2011), with some modifications, was used to determine the free radical scavenging activity of the *Lactobacillus* strains. The ABTS radical cation (ABTS°⁺) was prepared by reacting 7.4 mM ABTS and 2.6 mM potassium persulfate in equal quantities (1:1, v:v) for 12 h at room temperature in the dark, and diluting the mixture in methanol to obtain an absorbance of 1.1 ± 02 at 734 nm using a spectrophotometer (Barnstead International, USA). An amount of 200 µl of ABTS°⁺ solution was added to 50 µl of intact cells or intracellular cell-free extract of each *Lactobacillus* strain. After incubation at room temperature for 5 min in the dark, the absorbance was measured at 734 nm.

For both the FRAP and ABTS methods, the antioxidant activity was expressed as µg Trolox/ml sample solution (Trolox Equivalent Antioxidant Capacity), obtained using a standard curve prepared with Trolox.

2.2.3.3 Statistical analysis

The assays were carried out in two independent experiments, each in triplicate. The data were analysed as a completely randomized design (CRD) using the general linear model (GLM) procedure of SAS 9.2 (STATSOFT, 2008), with a model that included treatment effects and experimental error. All multiple comparisons amongst the means were carried out using Duncan's new multiple range test ($\alpha = 0.05$).

■ 3 Results and discussion

In the present study, two *Lactobacillus* strains were investigated for their antiproliferative (cytotoxic) effects against two cancer cell lines, and the antioxidant activities of the strains were also investigated, both using *in vitro* methods.

Since the selective induction of cancer cell death is a crucial factor for every anticancer agent (DAMIA; BROGGINI, 2004), in this study, the Chang cell line was used as a non-cancerous cell line to determine whether the two *Lactobacillus* strains were able to selectively kill the cancer cells without having adverse effects on the normal cells. The MTT assay was used to investigate the antiproliferation effects of two *Lactobacillus* strains. In the MTT assay, viable cells are able to reduce MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole] to a purple formazan product. After dissolution of the formazan product with a dissolution solution, the absorbance was recorded at an appropriate wavelength.

Figures 1, 2 and 3 show the results of the MTT assay for *L. pentosus* ITA23 against MDA-MB-231, HepG2 and the Chang cell lines, respectively. The supernatant of this strain exhibited good cytotoxic effects against both cancer
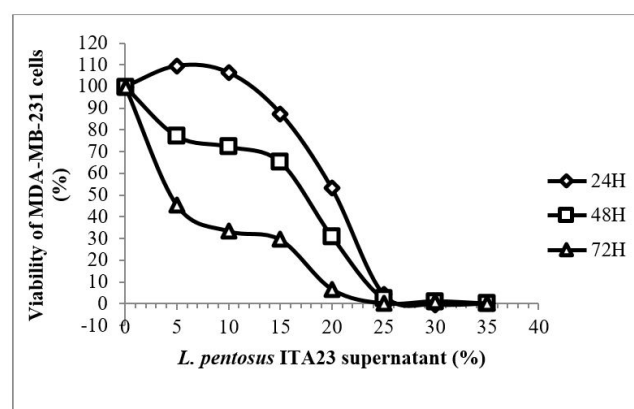

Figure 1. Cytotoxic effects of the *L. pentosus* ITA23 supernatants against the MDA-MB-231 cell line. The results are mean values from two independent experiments, each with three replicates.

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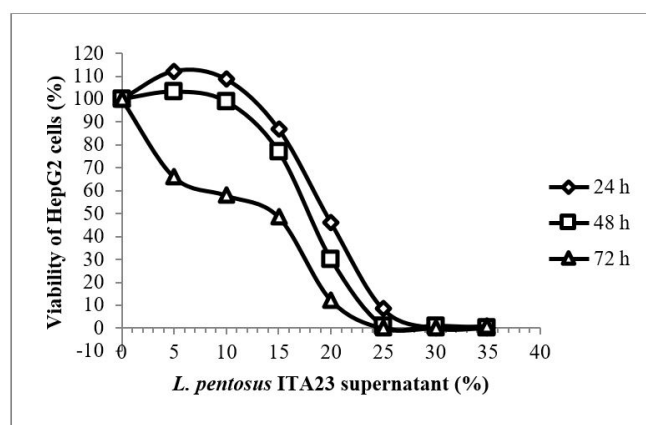*Shokryazdan, P. et al.*

Figure 2. Cytotoxic effects of the *L. pentosus* ITA23 supernatants against the HepG2 cell line. The results are mean values from two independent experiments, each with three replicates.
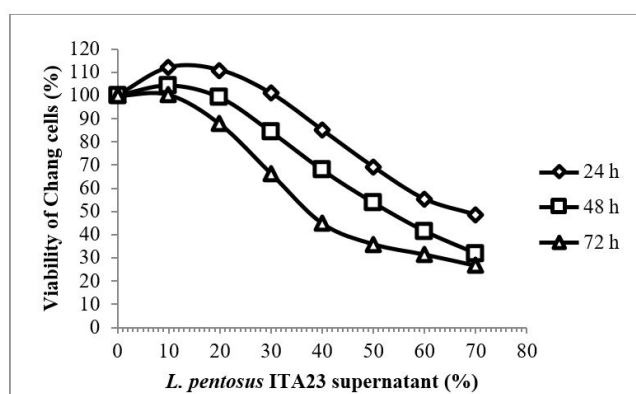

Figure 3. Cytotoxic effects of the *L. pentosus* ITA23 supernatants against the Chang cell line. The results are mean values from two independent experiments, each with three replicates.

Figure 4. Cytotoxic effects of the *L. acidipiscis* ITA44 supernatants against the MDA-MB-231 cell line. The results are mean values from two independent experiments, each with three replicates.

Figure 5. Cytotoxic effects of the *L. acidipiscis* ITA44 supernatants against the HepG2 cell line. The results are mean values from two independent experiments, each with three replicates.

Figure 6. Cytotoxic effects of the *L. acidipiscis* ITA44 supernatants against the Chang cell line. The results are mean values from two independent experiments, each with three replicates.

cell lines and was able to greatly inhibit their proliferation. At the three incubation times (24, 48 and 72 h of incubation), 27% of *L. pentosus* ITA23 supernatant was enough to completely kill the MDA-MB-231 cells (Figure 1). However, for the HepG2 cell line, the amount required was 30% at 24h and 25% at 48 and 72 h of incubation (Figure 2). On the other hand, the supernatant of *L. pentosus* ITA23 showed a lower antiproliferative effect against the normal cell line (Chang) in comparison with the cancer cell lines, since even the highest concentration tested (70%) for the longest incubation time (72 h), did not kill all the normal cells, only 73% of them (Figure 3).

The results of the MTT assay for *L. acidipiscis* ITA44 against the MDA-MB-231, HepG2 and Chang cell lines are presented in Figures 4, 5 and 6, respectively. *L. acidipiscis* ITA44, like *L. pentosus* ITA23, exhibited strong cytotoxic effects against both cancer cell lines. For both the MDA-MB-231 and HepG2 cell lines, 20% of *L. acidipiscis* ITA44 supernatant revealed 0% viability of the cancer cells after 24 and 48 h of incubation, and after 72 h of incubation, just 16% of the supernatant was enough to kill all the cancer cells. However,
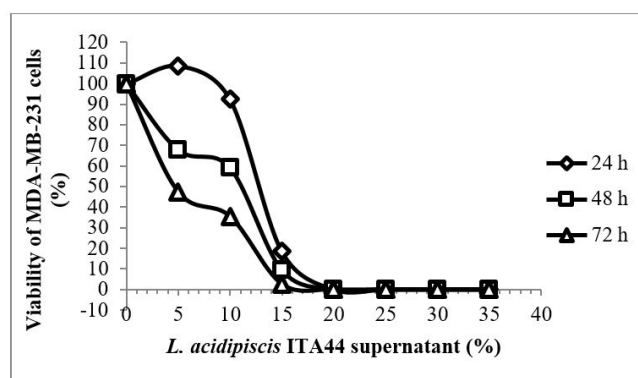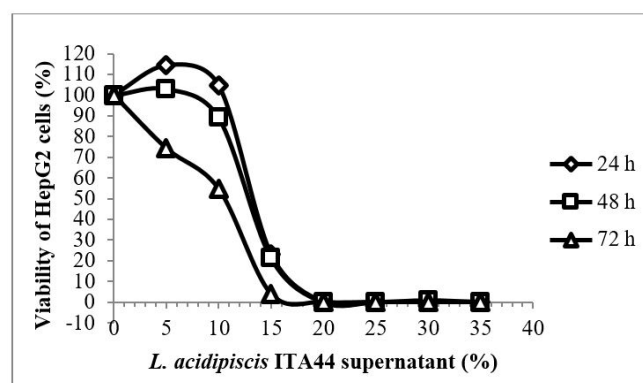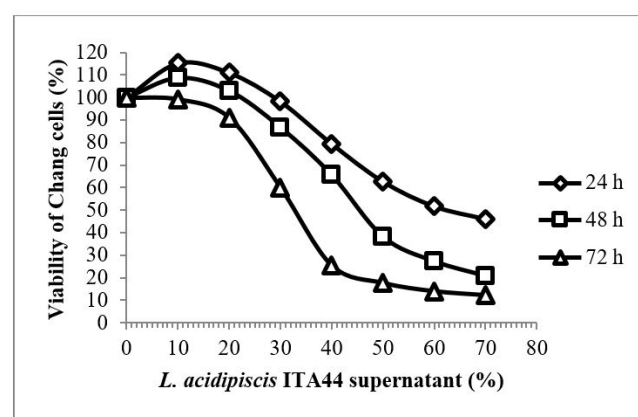
for the normal cells, the antiproliferative effect of the *L. acidipiscis* ITA44 supernatant was weaker than its effect on the cancer cells. Like the *L. pentosus* ITA23 supernatant, the *L. acidipiscis* ITA44 supernatant did not kill all the normal cells even at the highest concentration (70%) and the longest incubation time (72 h).

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Table 1 shows the results of the MTT assay in the form of the IC_{50}, which is the concentration of the *Lactobacillus* supernatant that causes a 50% reduction in cell viability. A lower value for the IC_{50} means greater efficacy of the *Lactobacillus* supernatant to inhibit proliferation of the cancer cells and vice versa. With regards to the IC_{50} values, it is clear that the supernatants of both *L. pentosus* ITA23 and *L. acidipiscis* ITA44 exhibited stronger antiproliferation effects against the cancer cell lines than against the normal cell line, because the IC_{50}s of the *Lactobacillus* supernatants on the normal cells were about three to eight times greater than those on the cancer cells. This means that both *L. pentosus* ITA23 and *L. acidipiscis* ITA44 were able to selectively kill the cancer cells.

The results of the MTT assay in the form of IC_{50} (Table 1) also showed that, as expected, longer incubation periods caused more inhibition of proliferation than shorter incubation periods for all the cell lines tested. For example, in the case of *L. pentosus* ITA23, the IC_{50}s of the supernatants on the MDA-MB-231 and Chang cells were 20.7 and 66.8% for 24 h, 17.5 and 52.7% for 48 h, and 4.7 and 37.2% for 72 h of incubation, respectively, showing that, for each cell line, a longer incubation time led to significantly ($p < 0.01$) smaller $IC_{50 values}$ than the shorter incubation times. For the HepG2 cell line, an incubation period of 72 h also showed greater ($p < 0.01$) effectiveness on the inhibition of proliferation of the cells than 24 and 48 h, since the corresponding IC_{50}s were 18.9, 18.0 and 14.8% after 24, 48 and 72 h of incubation, respectively. In the case of *L. acidipiscis* ITA44, the IC_{50}s values of the supernatants on MDA-MB-231 cells were significantly ($p < 0.01$) different from each other (13.2, 11.3 and 4.8% for 24, 48 and 72 h of incubation, respectively). The same trend of significant ($p < 0.01$) differences was observed for the Chang cells (61.2, 45.0 and 33.0% for 24, 48 and 72 h of incubation, respectively). The IC_{50}s values for the supernatants on the HepG2 after 72 h of incubation (10.5%) were also significantly ($p < 0.01$) higher than those after 24 and 48 h (13.6 and 13.3%, respectively).

Antiproliferative effects of bacterial strains on different cancer cell lines have been reported before (HAZA et al., 2004; TUO et al., 2010; BIFFI et al., 1997; THIRABUNYANON et al., 2009; CHOI et al., 2006). For example, Choi et al. (2006) investigated seven strains of *L. acidophilus*, *L. casei*, *L. brevis* and *L. rhamnosus* for their antiproliferative effects on HeLa, MCF7, U-87, HepG2, U2OS and PANC-1 cancer cell lines. They used the heat-killed cells as the treatments in their experiments and found that, of all the strains tested, *L. acidophilus* 606 (with 21-28% survival of cancer cells as compared to the control) and *L. casei* ATCC 393 (with 15-20% survival of cancer cells as compared to the control) exhibited the greatest inhibition effects on most of the cancer cells tested. In another study, Tuo et al. (2010) tested heat-killed bacterial cells, genomic DNA, and the cell walls of seven wild strains of *L. rhamnosus* and *L. paracasei*, isolated from traditional fermented foods, for their cytotoxic effects on the HT-29 cancer cell line. All the three fractions of the seven *Lactobacillus* strains exhibited direct antiproliferative activities against HT-29 cells. Of all the strains tested in their study, *L. coryniformis* ssp. *torquens* T3L exerted marked antiproliferative activities against the HT-29 cell line, with maximum inhibition rates of 30, 44.9, and 35.9% by its heat-killed bacterial cells, cell wall and genomic DNA, respectively. Haghshenas et al. (2014) also used some metabolites secreted by *Enterococcus* strains in the MTT assay against different cancer (MCF-7, HeLa, HT29, and AGS) and normal (HUVEC) human cell lines. They found that the metabolites tested had antiproliferative affects against cancer cells, but did not exhibit cell toxicity on the normal cell line. In another study, Desrouillères et al. (2015) used specific probiotic fermented milk components and cell walls extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 on cancer-induced rats. Based on the results obtained, they concluded that the probiotic bacteria and their metabolites released during the fermentation process could prevent colorectal carcinogenesis. Saxami et al. (2016) also reported a significant reduction in the proliferation of cancer cells by using both heated and non-heated conditioned media of *L. pentosus* B281 and *L. plantarum* B282. The results for the antiproliferative activity of the two *Lactobacillus*

Table 1. IC_{50}s (%) of the *Lactobacillus* strain supernatants on the different cell lines.

Lactobacillus strain	Incubation time (h)	Cell line		
		MDA-MB-231	**HepG2**	**Chang**
L. pentosus ITA23	24	20.7 ± 0.2^a	18.9 ± 0.3^a	66.6 ± 0.3^a
	48	17.5 ± 0.5^b	18.0 ± 0.5^a	52.7 ± 0.6^b
	72	4.7 ± 0.3^c	14.8 ± 0.4^b	37.2 ± 0.8^c
L. acidipiscis ITA44	24	13.2 ± 0.4^a	13.6 ± 0.2^a	61.2 ± 0.3^a
	48	11.3 ± 0.3^b	13.3 ± 0.3^a	45.0 ± 0.5^b
	72	4.8 ± 0.3^c	10.5 ± 0.5^b	33.0 ± 0.5^c

Results are mean values from two independent experiments, each in triplicate; [a-c]For each *Lactobacillus* strain, means within a column with different superscripts are significantly different (P < 0.01); MDA-MB-231, breast cancer cell line; HepG2, liver cancer cell line; Chang, normal liver cell line.

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strains used in the present study are consistent with the above-mentioned studies.

The mechanism of antiproliferative activity of *Lactobacillus* strains against cancer cells is still unclear. However, it may be attributed to the production of organic acids by the *Lactobacillus* strains and/or their antioxidant activity. Since some studies have indicated that different antioxidant compounds may have cytotoxic effects on different cancers (KATIYAR; MUKHTAR, 1997; LAMSON; BRIGNALL, 1999; SLAGA; BRACKEN, 1977), *Lactobacillus* strains which produce antioxidant compounds may have anti-carcinogenic effects against some types of cancer.

In the present study the total antioxidant activity and radical scavenging activity of the *Lactobacillus* strains was investigated using the FRAP and ABTS methods, respectively. Both intact cells and cell-free extract sections of the *Lactobacillus* cultures were used for the antioxidant assay, due to the fact that probiotic cells may either stay alive and intact in the intestinal tract of the host, or be lysed and release their intracellular extracts into the intestinal environment.

The results revealed that the two *Lactobacillus* strains tested showed antioxidant activity using both the FRAP and ABTS methods, especially by their intact cells when compared to the intracellular cell-free extracts (Figures 7 and 8). Using the FRAP method (Figure 7), *L. pentosus* ITA23 showed 135.28 and 23.91 µg trolox/ml antioxidant activity by its intact cells and cell-free extracts, respectively. The other *Lactobacillus* strain, *L. acidipiscis* ITA44, also showed similar results of 136.96 and 25.58 µg trolox/ml antioxidant activity by its intact cells and cell-free extracts, respectively. Using the ABTS method (Figure 8), *L. pentosus* ITA23 and *L. acidipiscis* ITA44 showed 166.34 and 144.26 µg trolox/ml antioxidant activity by their intact cells, respectively, and 39.11 and 45.6 µg trolox/ml antioxidant activity by their cell-free extracts.

Since the antioxidant activity of lactic acid bacteria is a new approach, there are not too many reports on the antioxidant activity of lactobacilli. In addition, the variation in the expression of the results and experimental conditions led to some difficulties in comparing the results of different experiments. In 2009, Klayraung and Okonogi (2009) investigated the antioxidant activity of two *L. fermentum* strains, which were isolated from fermented tea-leaves, using both the ABTS and FRAP methods. They reported Trolox Equivalent Antioxidant Capacities of 22.54 and 24.09 µM/ml supernatant for the two *L. fermentum* strains using the ABTS method, but using the FRAP method, these amounts were 20.63 and 21.26 µM/ml supernatant.

In the present study, the antioxidant activity of the intact cells of each of the *Lactobacillus* strains was higher than that of the intracellular cell-free extracts for both the
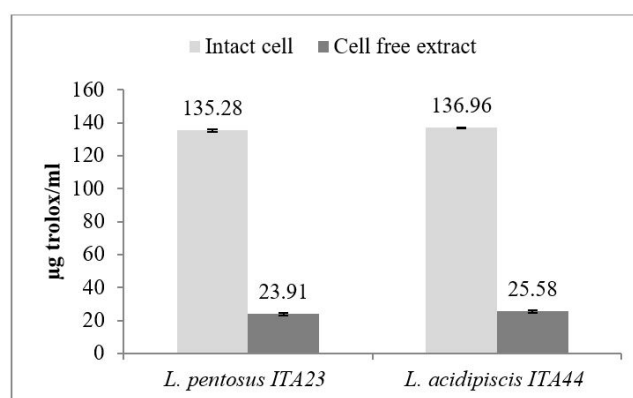

Figure 7. Antioxidant activity of the two *Lactobacillus* strains as measured by the FRAP assay. The results are mean values from two independent experiments, each with three replicates.
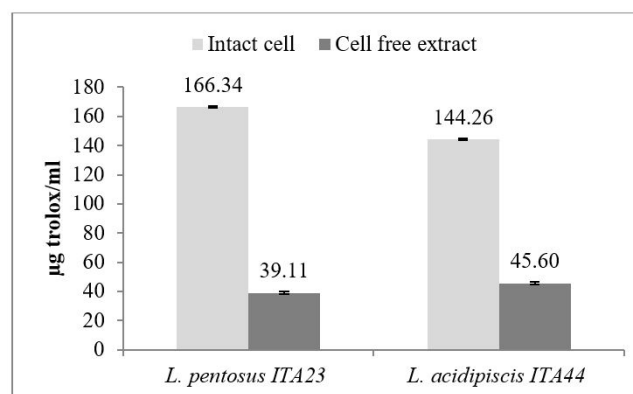

Figure 8. Antioxidant activity of the two *Lactobacillus* strains as measured by the ABTS assay. The results are mean values from two independent experiments, each with three replicates.

FRAP and ABTS methods. This could be attributed to the peptidoglycan and exopolysaccharide of the *Lactobacillus* cells, since the antioxidant activity of these fractions of the lactic acid bacteria have already been reported (LIU et al., 2011; CHABOT et al., 2001), although more studies are required to confirm this hypothesis.

In conclusion, both the *L. pentosus* ITA23 and *L. acidipiscis* ITA44 strains used in this study, which exhibited 100% killing of the cancer cells by, at the most, 30% of their supernatant, were considered to have remarkable antiproliferative effects against cancer cells. In addition, they showed specific selectivity in killing cancer cells and not normal cells. Furthermore, the two strains tested showed good antioxidant activity, especially by their intact cells. Hence, the two *Lactobacillus* strains could be considered as good potential probiotics for human consumption, due to their beneficial antioxidant and anticancer effects. Further investigations on their safety have to be carried out before they can be used in the food industry as probiotic strains.

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