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Data from literature suggest the possible use of probiotics as chemopreventive agents against colon cancer, but few investigations are available on their effects on gastric cancer proliferation. In our previous study, a specific *Lactobacillus,* strain *L. paracasei* IMPC2.1, was demonstrated to colonize the human gut and positively affect fecal bacteria and biochemical parameters. The aims of the present study were to investigate the effects of *L. paracasei* IMPC2.1, comparing them with those of *Lactobacillus rhamnosus GG* (*L.GG*), either as viable or heat-killed cells, on cell proliferation and apoptosis in a gastric cancer (HGC-27) and a colorectal cancer cell line (DLD-1). Both the gastric and colon cancer cells were sensitive to the growth inhibition and apoptosis induction by both viable or heat-killed cells from *L. paracasei* IMPC2.1 and *L.GG*. These findings suggest the possibility for a food supplement, based on dead probiotics, including *L. paracasei* IMPC2.1 cells, which could represent an effective component of a functional food strategy for cancer growth inhibition, with potential for cancer prevention.

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INTRODUCTION

Cancers of the gastrointestinal (GI) tract account for 25% of all neoplasms and for 9% of deaths from all causes in the world. Gastric and colon cancers remain the leading cause of cancer mortality throughout the world (1,2).

Many strategies have been proposed for preventing GI cancers: among them, surveillance of colonic polyps and chemoprevention. Chemoprevention can be described as all the treatments, nutritional or pharmacologic, that prevent, arrest, or regress the neoplastic growth in one or more organs (3).

Probiotics, defined as "dietary supplements, containing viable nonpathogenic micro-organisms, which confer health benefits to the host" (4), are considered potential chemopreventive agents, which are able to reduce the cancer risk by several mechanisms. These include binding and degradation of potential carcinogens; quantitative, qualitative, and metabolic

alterations of the intestinal microflora; production of antitumorigenic or antimutagenic compounds; enhancement of the host's immune response; and effects on host physiology (5,6). In addition, they can exert a key role in preventing cancer initiation and progression (7) as well as in controlling cell growth mechanisms (8).

Most probiotics belong to the genus *Lactobacillus*, which is a species with wide distribution in the GI tract (9). These strains are beneficial micro-organisms, which have been associated with several probiotic effects in both humans and animals (10). In spite of the large amount of data about their protective role in preventing colon cancer, few data are available on the potential of lactobacilli in interfering with the neoplastic transformation of gastric mucosa, because the stomach, which contains hydrochloric acid, is generally considered to be a hostile environment for bacteria. In this context, available results are mainly concerned with the preneoplastic condition of gastric mucosa due to *H. pylori* (*Hp*) infection, in which antibacterial and antiinflammatory effects have been shown by some lactobacilli such as *Lactobacillus brevis* (11), *Lactobacillus reuteri* (12), and *Lactobacillus rhamnosus GG (L.GG)* (13).

Our previous in vitro studies on a gastric cancer cell line originating from undifferentiated carcinoma of the stomach (HGC-27) established that administration of *L.GG* homogenate and cytoplasmic extracts affected the cell proliferation rates and reduced the cell polyamine content (14–16).

Among a plethora of available lactobacilli strains, the *L. paracasei* has been shown to have promising properties in the prevention and treatment of different GI diseases (17). Additional studies have also focused on the ability of *L. paracasei*, strain *L. paracasei* IMPC2.1, to influence the activity of intestinal microflora, the modulation of the immune system, as well as to inhibit different pathogens such as *Y. enterocolitica* (18–20).

The aims of our current study were 1) to investigate in vitro the antineoplastic activity of *L. paracasei* IMPC2.1 in comparison with that of *L.GG*, a strain well known for its probiotic characteristics, in the gastric cancer cell line HGC-27; 2) to compare these activities with those exerted on a colorectal cancer cell line (DLD-1); and 3) to compare the effects exerted by both viable and heat-killed lactobacilli on cell proliferation and apoptosis.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Two bacterial strains were tested: *Lactobacillus paracasei* IMPC2.1 (deposited as strain LMG P-22043 in the Belgian Coordinated Collections of Microorganisms, Ghent, Belgium) (21) and *Lactobacillus rhamnosus ATCC 53103* (commercially named *Lactobacillus GG*, obtained from the American Type Culture Collection ATCC, Manassas, VA).

L.GG and *L. paracasei* IMPC2.1 were incubated in deMan, Rogosa, and Sharpe (MRS) broth at 37°C overnight; the incubate was centrifuged at room temperature and the precipitate was collected and washed twice with phosphate-buffered solution (PBS) at pH 7.4. The bacteria were then resuspended in nonsupplemented Dulbecco Modified Eagle Medium (DMEM) for HGC-27 and in RPMI-1640 medium for DLD-1, to give a bacterial concentration of 10^8 CFU/ml. Heat-treatment of lactobacilli was performed by heating at 95°C for 1 h.

Human gastric cancer cell line HGC-27 and colon cancer cell line DLD-1 were obtained from the Interlab Cell Line Collection (Genoa, Italy). Cells were routinely cultured in DMEM and RPMI-1640 medium, respectively, supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, in a monolayer culture, and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. At confluence, the cells were harvested by trypsinization and serially subcultured with a 1:4 split ratio. All cell culture components were purchased from Sigma-Aldrich (Milan, Italy).

Acid Tolerance Assay

For the acid tolerance assay, bacteria were incubated in MRS broth at 37°C for 18 h, and 1 ml of culture was transferred into 9 ml PBS adjusted to pH 2.5 with 5M HCl and then incubated at 37°C for 3 h. The numbers of viable bacteria were determined at 0 h and 3 h of incubation, on an MRS agar plate. Triplicates of each sample were examined.

Adhesion of *L. paracasei* IMPC2.1 and *L.GG* to HGC-27 and DLD-1 Cells

HGC-27 and DLD-1 cells were seeded at a density of 2×10^5 cells/5 ml of DMEM and RPMI-1640, respectively containing 10% FBS in 60 mm tissue culture dishes (Corning Costar Co., Milan, Italy) and incubated for 48 h. Bacteria from 18-h cultures in MRS broth were harvested and washed twice with PBS. These bacteria were resuspended in nonsupplemented DMEM for HGC-27 adhesion assay and in RPMI-1640 medium for DLD-1 cell assay, to give a final concentration equal to 10^8 CFU/ml. After washing the HGC-27 and DLD-1 cells twice with PBS, a bacterial suspension (1 ml) was added to each plate and incubated at 37°C for 1 h and 3 h in 5% CO_2. Unattached bacteria were removed by washing with PBS 3 times. The cells were lysed with 0.1% Triton X-100 for 5 min. Adherent bacteria cells were counted in triplicate plates with MRS agar and then incubated at 37°C for 48 h.

Lactobacilli Treatment

In the experiments investigating the effects of *L. paracasei* IMPC2.1 and *L.GG* on cell proliferation and apoptosis, HGC-27 and DLD-1 cells (25th–30th passage) were seeded at a density of 2×10^5 cells/5 ml of DMEM and RPMI-1640 respectively, containing 10% FBS in 60-mm tissue culture dishes (Corning Costar Co., Milan, Italy). After 24 h, to allow for attachment, the medium was removed, and lactobacilli suspension containing viable cells (VCs) or heat-killed cells (HKCs) (10^8 CFU/ml) were added to cells.

Assessment of Cell Proliferation

After HGC-27 and DLD-1 cells had been cultured for 24 h or 48 h with lactobacilli suspensions containing VCs or HKCs (10^8 CFU/ml), the proliferative response was measured by colorimetric 3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and flow cytometry analysis.

To determine cell growth by colorimetric test, MTT stock solution (5 mg/ml in medium) was added to each dish at a volume of one-tenth the original culture volume and incubated for 2 h at 37°C in humidified CO_2. At the end of the incubation period, the medium was removed, and the blue formazan crystals were solubilized with acidic isopropanol (0.1 N HCl absolute isopropanol). MTT conversion to formazan by metabolically viable cells was monitored by spectrophotometer at an optical density of 570 nm.

The absolute number (cells/μl) of cultured cells was also determined by flow cytometry analysis comparing cellular events to bead events. For manual data analysis CellQuest software was used, dividing the number of cellular events by the number of bead events, and then multiplying by the TruCOUNT (BD Biosciences Europe, Erembodegem, Belgium) bead concentration. All the experiments were performed 4 times.

Apoptotic Cell Death

After HGC-27 and DLD-1 cells had been cultured for 24 h or 48 h with VCs and HKCs of *L. paracasei* IMPC2.1 and *L.GG*, the cytosolic DNA-histone complexes generated during apoptotic DNA fragmentation were evaluated by a cell death detection enzyme-linked immunoabsorbent assay (ELISA) kit (Roche Diagnostics GmbH, Mannheim, Germany) following the supplier's instructions. Each experiment was performed 3 times.

An additional method applied for apoptotic cell death evaluation was based on the use of flow cytometry analysis. HGC-27 and DLD-1 cells were administered with VCs and HKCs *L. paracasei* IMPC2.1 and *L.GG* for 24 h or 48 h, and the process was evaluated by Annexin V-FITC/7-AAD Kit (Immunotech, Marseille, France).

Briefly, cell suspensions were washed with PBS and centrifuged for 5 min at 500 × g at 4°C. Supernatants were discarded and cell pellets were resuspended in ice-cold Binding Buffer 1X to 5×10^6–10×10^6 cells/ml. Ten μl of Annexin V-FITC solution and 20 μl of 7-AAD Viability Dye were added to 100 μl of each cell suspension and mixed gently. The tubes were kept on ice and incubated for 15 min in the dark. Then, 100 μl of ice-cold Binding Buffer 1X were added and mixed gently.

For each cell preparation, acquisition and analysis were done on a FacsCalibur® equipped with the CellQuest™ software. The biparametric dotplot LOG FL1 vs. LOG FL2 shows 3 distinct populations: 1) the viable cells, which have low FITC and a low 7-AAD signal; 2) the apoptotic cells, which have high FITC and a low 7-AAD signal; and (3) the secondary necrotic cells, which have high FITC and a high 7-AAD signal.

Statistical Analysis

Because of the nonnormal distribution of the data, nonparametric tests were performed. All values were subjected to Arcsine transformation before analyses. For adhesion assay of *L. paracasei* IMPC2.1 and *L.GG*, the significance of differences between the 2 cell lines was analyzed with the Wilcoxon Mann Whitney test. For proliferative characteristics of HGC-27 and DLD-1 cells, the significance of differences between the groups was determined by the Kruskal Wallis analysis of variance. All data are expressed as median value (range). Differences were considered significant at $P < 0.05$. A specific software package (SigmaStat for Windows version 3.00 SPSS Inc. San Jose, CA) was used.

RESULTS

Survival and Adhesion of *L. paracasei* IMPC2.1 and *L.GG* to HGC-27 and DLD-1 Cells

Survival of *L. paracasei* IMPC2.1 and *L.GG* in a GI tract model is shown in Fig. 1A. After exposure to pH 2.5 for 3 h, both lactobacilli strains did not significantly reduce their

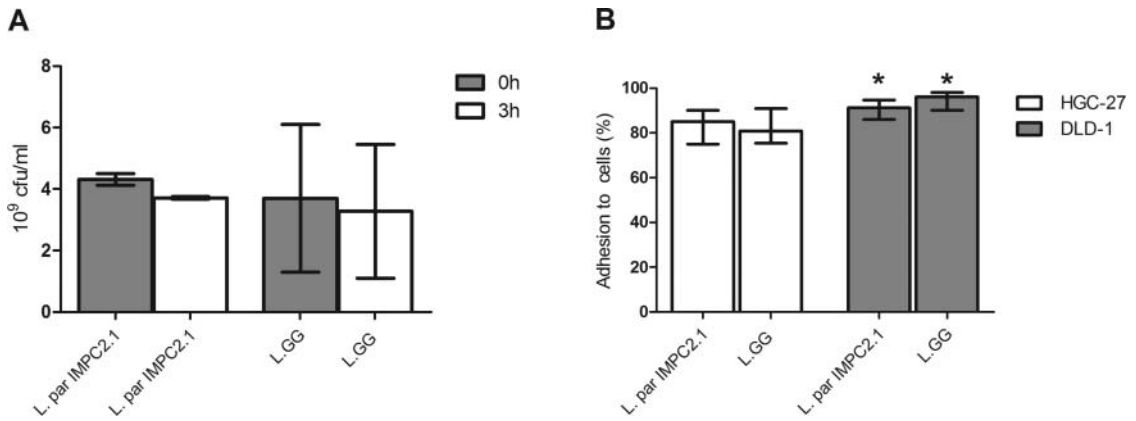


FIG. 1. A: Survival of *L. paracasei* IMPC2.1 and *L.GG* (expressed by cfu/ml) after exposure to pH 2.5 for 3 h. B: Percentage of adhesion of *L. paracasei* IMPC2.1 and *L.GG* to HGC-27 and DLD-1 cells after 1 h of incubation. Data are expressed as median and the range of three experiments. $^*P < 0.05$ Wilcoxon Mann Whitney test.

number (as expressed by cfu/ml). The initial concentration of *L. paracasei* IMPC2.1 was 4.3×10^9 cfu/ml (4.125×10^9–4.5×10^9); after 3 h of incubation it was equal to 3.7×10^9 cfu/ml (3.675×10^9–3.75×10^9). For *L.GG*, its initial value was 3.7×10^9 cfu/ml (1.3×10^9–6.1×10^9); after 3 h of exposure it was equal to 3.3×10^9 cfu/ml (1.1×10^9–5.45×10^9).

The efficiency of the lactobacilli strains in adhering to gastric and colonic cancer cells after 1 h of incubation is shown in Fig. 1B. The 2 strains of lactobacilli studied were able to adhere either to gastric (HGC-27) or colon (DLD-1) cancer cells and, in each cell line, no differences between their percentages of adhesion were present. In the gastric cancer cells, the percentages of adhered *L. paracasei* IMPC2.1 and *L.GG* were 85% (75%–90%) and 80.9% (75.4%–90.9%), respectively. In the colon cancer cells, the percentages of adhered lactobacilli were slightly higher being 91.2% (86%–94.7%) and 96% (90%–98%) for *L. paracasei* IMPC2.1 and *L.GG*, respectively. A cell line specificity was also observed, because *L. paracasei* IMPC2.1 and *L.GG* showed a significantly ($P < 0.05$) higher adhesion ability to DLD-1 cells than to HGC-27 cells. There were no differences related to bacterial strains, because both the probiotics showed the same capacity of adhesion to the 2 tested GI cell lines.

Effects of *L. paracasei* IMPC2.1 and *L.GG* on HGC-27 and DLD-1 Cell Proliferation

To determine whether *L. paracasei* IMPC2.1 and *L.GG* were able to inhibit the growth of the HGC-27 and DLD-1 cell lines, the 2 strains of lactobacilli (10^8 cfu/ml) were administered to the cell cultures as VCs and HKCs for 24 or 48 h. Both after 24 h and 48 h treatments, VCs and HKCs of the 2 probiotic strains caused a significant ($P < 0.01$) reduction in conversion of the MTT tetrazolium salt in HGC-27 and DLD-1 cells compared with the untreated control cells (Table 1).

The same significant proliferative response was also observed by flow cytometry analysis (Table 2). After 24 h and 48 h of treatment, VCs and HKCs of both *L. paracasei* IMPC2.1 and *L.GG* caused a significant ($P < 0.01$) decrease in HGC-27 and DLD-1 cell proliferation compared to control cells.

Interestingly in HGC-27 cells, 48 h of treatment with VCs and HKCs of the 2 lactobacilli strains caused a more marked reduction, although not significant, in the proliferation activity compared to 24 h treatment. By contrast, in DLD-1 cells the reduction after 48 h of treatment was evident only in *L. paracasei* IMPC2.1 treated cells, but not in *L.GG* ones, compared to 24 h.

Effects of *L. paracasei* IMPC2.1 and *L.GG* on HGC-27 and DLD-1 Apoptotic Cell Death

Figure 2 shows the effects of VCs and HKCs of *L. paracasei* IMPC2.1 and *L.GG* on the apoptosis of HGC-27 and DLD-1 cells, evaluated by ELISA.

After 24 h and 48 h, the exposition of HGC-27 and DLD-1 cells to VCs and HKCs of both *L. paracasei* IMPC2.1 and *L.GG* gave rise to a significant ($P < 0.01$) proapoptotic effect compared to control cells.

The apoptotic response of HGC-27 and DLD-1 cells to VCs and HKCs of *L. paracasei* IMPC2.1 and *L.GG* was further confirmed by flow cytometry analysis.

After 24 h and 48 h of treatment with VCs and HKCs of *L. paracasei* IMPC2.1 and *L.GG*, the percentages of HGC-27 (Fig. 3) and DLD-1 apoptotic cells (Fig. 4) were significantly increased compared to control cells ($P < 0.01$).

DISCUSSION

GI neoplasms represent a major public health problem and diet may make an important contribution to their risk (22), implying that it is potentially reducible. Evidence also supports

TABLE 1
Proliferation activity of HGC-27 cell line and DLD-1 cell line after 24 and 48 h of treatment with viable cells (VCs) and heat-killed cells (HKCs) of *L. GG* and *L. paracasei* as evaluated by MTT test

	24 h			48 h		
Cell line	Control	VCs	HKCs	Control	VCs	HKCs
HGC-27						
L. GG	100[a]	85.16[b] (80.77–100)	88.36[b] (80.5–100)	100[a]	73.25[b] (34.61–88.0)	80.5[b] (73.08–100)
L. paracasei	100[a]	89.9[b] (83.0–98.15)	81.45[b] (60.0–100)	100[a]	71.42[b] (51.72–100)	78.5[b] (55.17–100)
DLD–1						
L. GG	100[a]	72.27[b] (61.67–86.75)	83.06[b] (69.23–100)	100[a]	74.76[b] (61.36–100)	85.86[b] (73.91–95.0)
L. paracasei	100[a]	78.0[b] (58.46–100)	82.5[b] (69.23–100)	100[a]	61.1[b] (30.3–68.11)	76.51[b] (61.67–90.0)

Data are expressed as percentage of viable cells (median value and the range). Different letters differ significantly ($P < 0.01$, Kruskal Wallis analysis of variance).

TABLE 2
Proliferation activity of HGC-27 cell line (panel A) and DLD-1 cell line (panel B) after 24 and 48 hours of treatment with viable cells (VCs) and heat-killed cells (HKCs) of *L. GG* and *L. paracasei* as evaluated by flow cytometry analysis

	24 h			48 h		
Cell line	Control	VCs	HKCs	Control	VCs	HKCs
HGC-27						
L. GG	100[a]	94.15[b] (72.9–100)	95.05[b] (90.0–100)	100[a]	55.12[b] (35.44–100)	82.36[b] (73.65–97.69)
L. paracasei	100[a]	89.21[b] (70.79–100)	94.05[b] (49.88–100)	100[a]	77.75[b] (41.75–100)	79.7[b] (70.6–100)
DLD-1						
L. GG	100[a]	83.36[b] (72.95–100)	95.44[b] (70.0–100)	100[a]	87.58[b] (58.95–100)	96.9[b] (90.0–100)
L. paracasei	100[a]	88.95[b] (64.5–100)	91.41[b] (81.0–100)	100[a]	67.32[b] (61.98–89.29)	87.06[b] (60.21–95.39)

Data are expressed as percentage of viable cells (median value and the range). Different letters differ significantly ($P < 0.01$, Kruskal Wallis analysis of variance).

the view that the colonic microflora is involved in the etiology of these cancers, at least for those occurring in the large bowel. Therefore, dietary interventions and use of natural bioactive supplements to reduce the risks of neoplastic transformation, as putative chemopreventive substances, have been extensively studied. Postulated mechanisms include reduction in the activity of several cancer causing bacteria as well as dismutagenic and anti-carcinogenic actions (23).

Probiotics are live bacteria that could exert beneficial health effects upon consumption. Use of probiotic therapy has progressively increased for prevention and treatment of a number of GI disorders, including irritable bowel syndrome, inflammatory bowel disease, pathogenic bacterial or viral infection, and antibiotic-associated diarrhea (24,25). There is also epidemiological evidence that supports a protective role of probiotics against cancer (26).

Such anticarcinogenic properties have also been studied at a molecular level (27) and by analysis of intermediate biomarkers of proliferation. In our previous in vivo study, ingestion of VSL#3 reduced polyamine levels and ornithine decarboxylase (ODC) activity in colorectal mucosa of rats (7). ODC is involved in the biosynthesis of polyamines that are important in cell proliferation, differentiation, and macromolecular synthesis. Increased ODC activity has been associated with increasing colon adenomas and carcinomas, which indicates a hyper-proliferative state of the colonic mucosa (28).

Among probiotics, lactobacilli along with bifidobacteria, have probably been the best studied microorganisms. Data from literature indicate a possible use of these bacteria in the therapeutic inhibition of colon cancer development (29). By contrast, few investigations are available on the possible effects of lactobacilli on gastric cancer. Despite this, lactobacilli have been proven to be helpful in the management of a gastric pre-neoplastic condition, such as *Hp* infection (11).

One suggested mechanism related to probiotic therapy is that Lactobacilli spp. can adhere and even transiently reside in

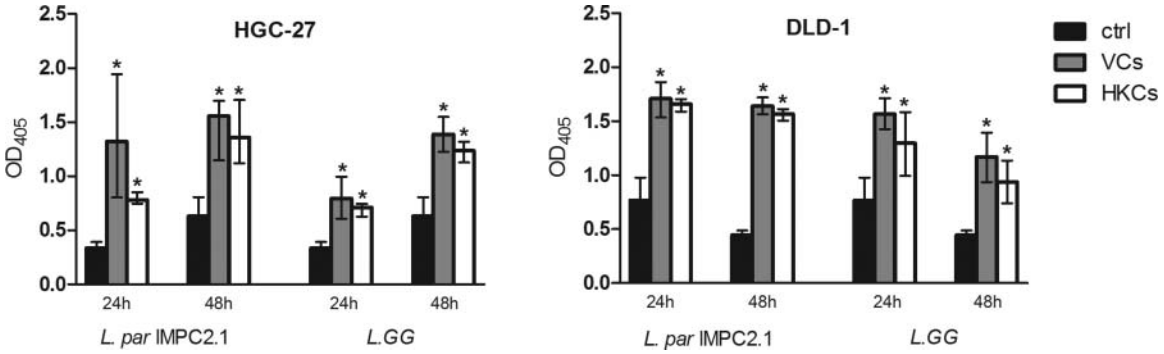
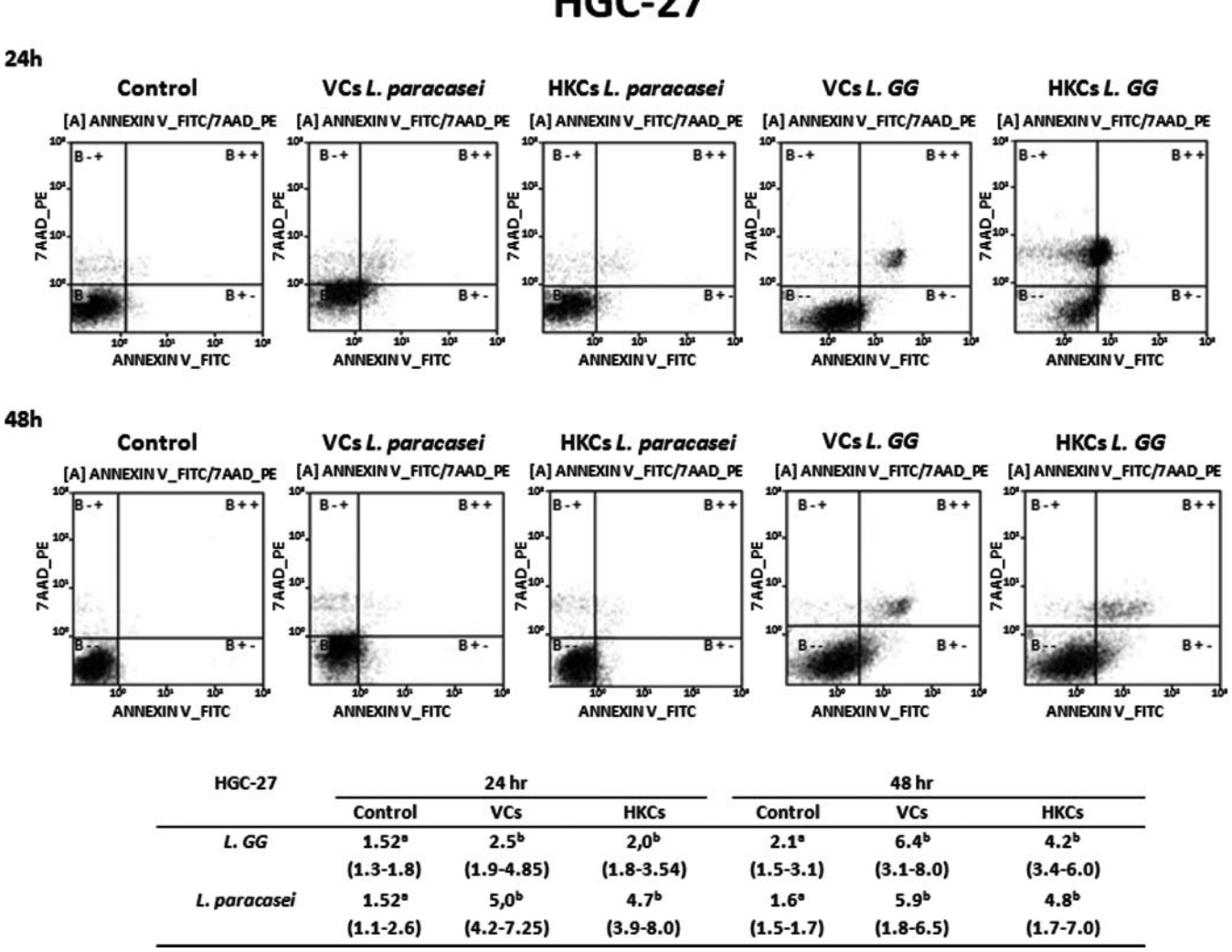


FIG. 2. The apoptosis induction of viable cells (VCs) and heat-killed cells (HKCs) of *L. paracasei* IMPC2.1 and *L.GG* on gastric cancer cells (HGC-27) (A) and colon cancer cells (DLD-1) (B) using Cell Death Elisa Kit after 24 h and 48 h of treatment. Black = control; Grey = VCs; White = HKCs. Data are expressed as median and the range of 3 experiments. $^*P < 0.01$ Kruskal Wallis analysis of variance.



HGC-27	24 hr			48 hr		
	Control	VCs	HKCs	Control	VCs	HKCs
L. GG	1.52[a]	2.5[b]	2,0[b]	2.1[a]	6.4[b]	4.2[b]
	(1.3-1.8)	(1.9-4.85)	(1.8-3.54)	(1.5-3.1)	(3.1-8.0)	(3.4-6.0)
L. paracasei	1.52[a]	5,0[b]	4.7[b]	1.6[a]	5.9[b]	4.8[b]
	(1.1-2.6)	(4.2-7.25)	(3.9-8.0)	(1.5-1.7)	(1.8-6.5)	(1.7-7.0)

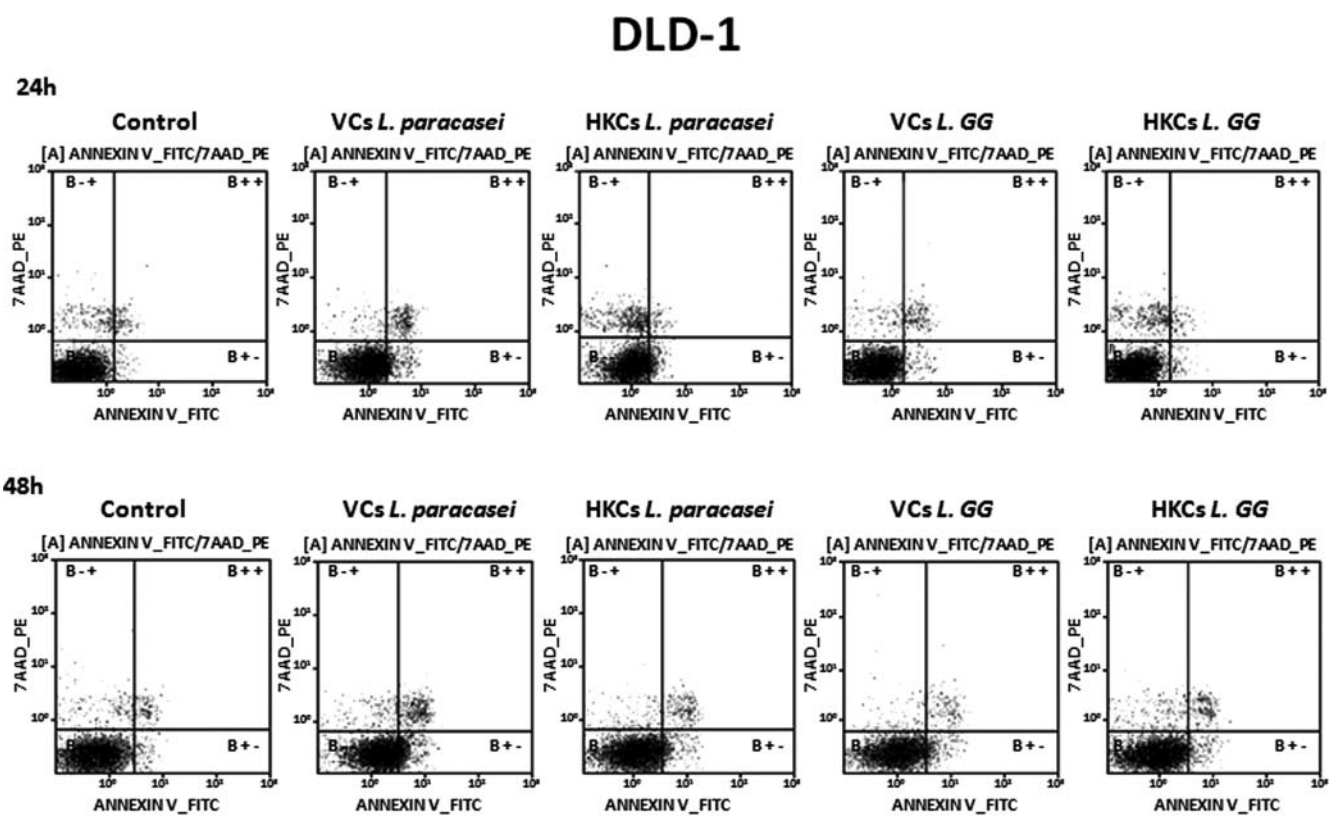
FIG. 3. Analysis of apoptosis in HGC-27 cells treated with viable cells (VCs) and heat-killed cells (HKCs) of *L. paracasei* IMPC2.1 and *L.GG* using flow cytometry analysis after 24 h and 48 h of treatment. Each cytogram shows 4 regions (B): B- contains the vital population, B+- contains the apoptotic cells, B++ contains the dead cells, B-+ contains the cells damaged during the scraping procedure. Data are expressed as percentage of apoptotic cells (median value and the range). Different letters differ significantly ($P < 0.01$ Kruskal Wallis analysis of variance).

the stomach, enhance the immune response, and reduce the *Hp* inflammation effect on the host gastric mucosa (30).

Along with these well-known co-adjuvant effects in *Hp* therapy, it could be interesting, from a chemopreventive point of view, to evaluate the capabilities of some lactobacilli in modulating the growth of gastric cancer cells. Among them, *L.GG* is a strain well-known for its probiotic characteristics (31), and it has already been shown in vitro to affect the cell growth and polyamine biosynthesis of both HGC-27 and DLD-1 human cancer cell lines (15,16). Interestingly, when the cytoplasmic and cell wall extracts derived from *L.GG* homogenate were tested, the cytoplasmic extract, but not the cell wall extract, was shown to be suppressive.

L. paracasei spp are also considered to be effective as probiotics since they possess abilities in modifying the metabolic activities of intestinal microflora and modulating the immune system (20,21). Experimental evidence for their anti-carcinogenic activity comes primarily from in vitro studies, although performed on cell lines other than GI ones (32). In this framework, an aim of this study was to explore in vitro the antiproliferative properties of a *L. paracasei* strain (*L. paracasei* IMPC2.1) and to compare them with those of *L.GG*, either as viable or heat-killed cells on cells from gastric and colorectal neoplasms.

The 2 strains were tested for their tolerance to low pH conditions and for their ability to adhere to GI cell lines. Tolerance to low pH is an important property for the survival of bacteria



DLD-1	24 hr			48 hr		
	Control	VCs	HKCs	Control	VCs	HKCs
L. GG	1.7[a]	4.8[b]	4.5[b]	0.45[a]	1.2[b]	1.15[b]
	(0.5-5.5)	(2.9-9.0)	(2.4-8.4)	(0.1-0.9)	(0.5-4.0)	(0.4-4.2)
L. paracasei	1.7[a]	5.65[b]	5.9[b]	0.45[a]	3.9[b]	3,0[b]
	(0.5-5.5)	(3.7-10)	(2.6-9.0)	(0.1-0.9)	(0.9-7.9)	(0.7-6.8)

FIG. 4. Analysis of apoptosis in DLD-1 cells treated with viable cells (VCs) and heat-killed cells (HKCs) of *L. paracasei* IMPC2.1 and *L.GG* using flow cytometry analysis after 24 h and 48 h of treatment. Each cytogram shows four regions (B): B– contains the vital population, B+- contains the apoptotic cells, B++ contains the dead cells, B-+ contains the cells damaged during the scraping procedure. Data are expressed as percentage of apoptotic cells (median value and the range). Different letters differ significantly ($P < 0.01$ Kruskal Wallis analysis of variance).

under the conditions in the stomach. Both strains demonstrated a remarkably high tolerance to pH 2.5 after 3 h of exposure. Corcoran et al. (33) reported that L. GG had a high survival rate over the 90 min of exposure to simulated gastric juice (pH 2.0). In a previous paper by Lavermicocca et al. (20) a survival test following 3 h of incubation at pH 2.0 demonstrated that only L. *paracasei* IMPC2.1 and L. *plantarum* ITM21b, but not *L.GG*, survived (values above the detection limit). However, survival following 4 h of incubation at pH 2.5 was observed for both L. *paracasei* IMPC2.1 and *L.GG* along with other lactobacilli strains. These data confirm that the survival rates of Lactobacillus species at low pH values differ and that differences are also apparent at the strain level.

In addition, adhesion to intestinal surfaces represents a major property for probiotic strains, because intestinal attachment is important for colonization of the GI tract for many bacterial species. Whatever the origin of the cancer cell lines, both the strains were able to adhere to the cell surface monolayer in our set of experiments. However, the significantly higher number of adherent bacteria was observed with the DLD-1 cell line compared to HGC-27 cells, with an average of 91% and 95% for *L. paracasei* IMPC2.1 and *L.GG* after 1 h of exposure, respectively. No major difference was observed between the levels of adhesion obtained after 1 h and 3 h of incubation, suggesting that adhesion occurs rapidly after the initial contact between the cells and the bacteria (data not shown).

A few papers are available on the adhesion activity of lactobacilli to colon cancer cell lines that report results as percentage values. These papers (34–36) report *L.GG* adhesion to CaCo-2 cells showing very low percentages of adhesion, ranging from 9.7% to 15.7%. Our values of adhesion seem particularly high when compared to those results. An explanation for these discrepancies may be provided by the different cell lines and strains that were investigated, the number of bacteria added, as well as the assay conditions (37).

Few data are available for the adhesion activity of lactobacilli to gastric cancer cells. Among them, 2 papers investigated the adhesion capacity of different lactobacilli to Kato III and MKN45 gastric cancer cell lines (38,39). Unfortunately, the level of adhesion was not expressed as percentage of adhesion; therefore these data are not comparable to our present results.

L. paracasei IMPC2.1 and *L.GG* were screened for their antiproliferative activity and promotion of apoptosis in neoplastic cells. *L. paracasei* IMPC2.1 showed antiproliferative activity quite similar to that exerted by *L.GG.* Interestingly, the 2 lactobacilli strains, either as viable or heat-killed cells, decreased proliferation significantly in both HGC-27 and DLD-1 cells after both 24 h and 48 h of culture, as demonstrated by the significant reduction in MTT conversion and flow cytometry analysis.

The rates of cell proliferation and apoptosis (programmed cell death) may determine the speed of neoplastic growth (40). Apoptosis is frequently impaired in many human tumors and is also an important phenomenon in chemotherapy-induced tumor cell death. Therefore, the modulation of apoptosis has been hypothesized as an effective technique in the treatment of cancer (41). HGC-27 and DLD-1 cells had a significant induction of apoptosis after treatment with either viable or heat-killed bacterial cells of both strains.

Overall, the antiproliferative effect and the induction of apoptosis caused by *L. paracasei* IMPC2.1 and *L.GG* were quite similar in both gastric and colon cancer cells and they were mediated not only by live microorganisms but also by nonviable ones. It is widely accepted that different fractions of lactobacilli, such as the whole cells, heat-killed cells, but also cell wall, peptidoglycan, and cytoplasmic fraction, may show a clear antiproliferative effects against human cancer cell lines (42).

It is unclear if the in vitro efficacy of these lactobacillus strains in adhering to DLD-1 and HGC-27 cell lines could be fully replicated in vivo in the GI tract. However, it could be a reliable indicator when selecting the probiotic strain.

It is beyond the scope of this article to identify the heat stable bacterial component that was effective in inhibiting cell proliferation, but our findings about the antiproliferative and proapoptotic activities of heat-killed lactobacilli are in agreement with other reports. Thus, there is still debate as to which substances are involved. Choi et al. (43) demonstrated that the soluble polysaccharides isolated from a lactobacillus such as *L. acidophilus 606*, but not proteins or lipids extracts, constitute the major fraction that inhibits the proliferation of various colon cancer cells. Ma et al. (44) however, reported that the antiproliferative effect of *Bacillus polyfermenticus* on various colon cancer cell lines was probably due to heat stable bacterial proteins. The positive effects shown by both live and dead probiotics support the probiotic paradox, in which both live and dead cells seem to be capable of generating a different biological response (45,46). Thus, live probiotic cells influence both the GI microflora and the immune response whilst the components of dead cells exert an antiinflammatory response in the GI tract. Furthermore, variable amounts of dead cells might contribute to the variation in response often seen with live probiotic cultures. The findings that live probiotics may not be mandatory to be beneficial could therefore have a major impact on the practical use and manufacturing of probiotics (47). Products based on dead cells would be relatively easy to standardize and would have a long shelf-life. These findings suggest the possibility for a food supplement, based on dead probiotics, including *L. paracasei* IMPC2.1 cells, which could represent an effective component of a functional food strategy for cancer growth inhibition, with potential for cancer prevention.

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