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PROBIOTIC STRAINS: SURVIVAL UNDER SIMULATED GASTROINTESTINAL CONDITIONS, IN VITRO ADHESION TO CACO-2 CELLS AND EFFECT ON CYTOKINE SECRETION

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Running title: Evaluation of probiotic strains

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Abstract

This study evaluated three probiotic strains (*Lactobacillus paracasei* subsp. *paracasei* LC-01, *L. acidophilus* LA-5, *Bifidobacterium lactis* Bb-12) and two yoghurt strains (*L. delbrueckii* subsp. *bulgaricus* LBY-27 and *Streptococcus thermophilus* STY-31) with regard to their resistance to simulated gastrointestinal stress, and their ability to interact with human intestinal epithelial cells. The viability of strains was analyzed by measurements of fluorescence-stained cells and their growth by plate colony-counts. The results reveal that for all tested strains, gastric emptying (above pH 3.0) would release a large number of viable cells ranging from 91% for *L. paracasei* to 53% for *S. thermophilus* into the intestinal tract, and that between 12%-23% of them subsequently survive intestinal stress. Among them *L. paracasei* showed the highest resistance to gastric stress. All the bacteria adhered to the Caco-2 cell line, with the highest adhesions being observed for *L. delbrueckii* subsp. *bulgaricus* (9%) and *L. acidophilus* (7%). Binding of all strains to Caco-2 cells did not result in a significant increase in the production of IL6 and IL8 cytokines, suggesting that these bacteria do not trigger an overt inflammatory response in human intestine epithelial cells.

Keywords: Probiotic bacteria; lactic acid bacteria, gastrointestinal stress, adhesion; immunomodulation.

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Introduction

Probiotics are defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit to the host [1]. There is clinical evidence that supports the health-promoting characteristics of *Lactobacillus* and *Bifidobacterium* spp, which are often included in fermented milk products [2]. However, the minimum amounts of probiotics needed to obtain a clinical effect have not been established. As more information on probiotics becomes available, it seems likely that these amounts will vary as a function of the strain and the health effect desired [3], and of the probiotic´s capability to display specific responses at various sites along the intestine [4]. Probiotic product specifications require strains to be designated individually, appropriately classified as to species, and retain an acceptable viable count at the end of their shelf life in the designated product formulation [1]. The CODEX standard for fermented milks [5] establishes that the minimum counts of these micro-organisms at the time of consumption should be 10^6 cfu g^{-1} [5]. In addition to their ability to survive in the product, many criteria have been suggested for the selection of probiotics, among them the tolerance of gastrointestinal conditions (acid and bile) and ability to adhere to intestinal mucosa. Bacterial viability is reduced along the entire tract, but it is apparent that the acidic environment of the stomach and the presence of bile in the duodenum are the major factors affecting viability [6]. *In vivo* studies are too complex to be used for high throughput screening of bacteria viability. Several studies have been reported by other authors about the viability cell after simulated gastrointestinal tract conditions [7, 8]. Therefore, several *in vitro* multi-compartmental models, which simulate different parts of the human gastrointestinal tract, have been developed to study the survival rate of potential probiotic strains. Among them Mainville et al. [9] recently developed a dynamic *in vitro* model simulating the events of food ingestion and digestion, allowing the addition of a food matrix before or along with the probiotic strain to be tested.

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Bacterial growth viability is typically assessed by plate counting. However there are a number of disadvantages associated with this approach, such as the relatively long time needed to form visible colonies, and the possible underestimation of viable micro-organisms, which do not form colonies because they are sublethally damaged, dormant (inactive but ultimately culturable) or active but non-culturable [10, 11]. To resolve these problems other technologies such as fluorescent detection of cells have been developed [12, 13]. Thus, the combined use of SYTO9 and propidium iodide fluorescent dyes allows the differential staining of the nucleic acids of intact cells and of those with compromised membranes, though there has been some debate as to the efficacy of these methods in complex matrices. Nevertheless, they have been successfully used for detection of probiotic bacteria [14; 12] and lactic acid bacteria (LAB) [15] in a food matrix.

Adhesion to intestinal mucosa is also regarded as a prerequisite for probiotic micro-organisms, allowing possible colonization of the intestinal tract [16]. The difficulties of studying bacterial adhesion *in vivo*, have led to the development of *in vitro* model systems for the preliminary studies of adherent strains [17]. These models are based on adhesion to tissue culture cell lines such as Caco-2 and HT-29, which differentiate and closely resemble the enterocytes of human small intestine, and to human intestinal mucus [18]. Adhesion is affected by many factors such resident flora in the gastrointestinal tract, pH, growth phase of the bacteria, density of the bacterial suspension, intensity of washing out the unbound cells, etc. [19]. Furthermore, the adhesion capacity may be correlated with transient colonization of intestinal cells, which could be a factor for their immunomodulation by probiotics [20]. It is well known that one of the potential benefits of probiotic therapy is the suppression of the inflammatory process [21, 22]. The secretion of the pro-inflammatory cytokines such as IL-6 and IL8 is therefore a hallmark of the inflammatory response in the intestine [23].

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In this study, the viability of various probiotic and LAB strains in acidified milk was assessed after exposing the cells *in vitro* to gastric, or to gastrointestinal, stress conditions. Moreover, human intestinal epithelial-like Caco-2 cells were used to examine the adhesion of the bacteria and their ability to stimulate pro-inflammatory cytokine production in this cell line.

Materials and methods

Microorganisms, growth conditions and preparation of milk-cell suspension

The bacterial strains used were *Lactobacillus paracasei* subsp. *paracasei* LC-01, *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* Bb-12, *Lactobacillus delbrueckii* subsp. *bulgaricus* LBY-27 and *Streptococcus thermophilus* STY-31. The strains were isolated from a commercial synbiotic product (Synbiotic Drink; Priégola, Madrid, Spain) and identified by molecular typing described previously [24]. Lactobacilli and bifidobacteria strains were propagated on MRS broth (Pronadisa, Madrid, Spain). The medium was supplemented with 0.05 % L-cysteine hydrochloride (Merck, Darmstad, Germany) for all bacteria (except for *L. paracasei*) and supplemented with 0.1% Tween for *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* growth. *S. thermophilus* was grown in ETSY medium (Pronadisa) containing 0.5% lactose. All incubations were performed at 37 ºC except for *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, which were grown at 42 ºC. *B. lactis*. *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* were grown anaerobically in jars (AnaeroGen™, Oxoid Unipath Ltd. Basingstoke, Hampshire, UK).

For preparation of milk-cell suspension, the strains were grown in fresh medium until they reached the late exponential phase (approximately 10^9 cfu mL^{-1} for *L. paracasei, B. lactis*, and *S. thermophilus*; 10^8 cfu mL^{-1} for *L. delbrueckii* subsp. *bulgaricus* and 10^7 cfu mL^{-1} for *L. acidophilus*). Cells from 25 mL of culture were

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sedimented by centrifugation, 10,000 × g for 10 min, and resuspended in the same volume of 10% reconstituted skim milk powder (autoclaved 110 ºC, 15 min) acidified with 1 M HCl (Merck) to pH 4.6. (corresponding to the pH of the commercial synbiotic drink)

For adhesion experiments, bacteria were grown until they reached the late exponential phase and they were sedimented as described above. Then, they were resuspended in the appropriate volume of Dulbecco´s modified Eagle medium (DMEM, Invitrogen) to give 1.25×10^6 cfu mL^{-1}.

Gastric and gastrointestinal transit tolerance assay

The gastrointestinal tract model is depicted schematically in Fig. 1. The experiments were performed in triplicate; three independent cultures of each bacterium were analyzed as follows. Milk-cell suspensions were prepared as described above and samples of 2.5 mL were withdrawn prior (sample G1, untreated control) or after (samples G2 through G7) the indicated treatments to determine cell survival by the tests described below. The gastric and gastrointestinal solutions were prepared fresh daily according to the protocols described by Marteau et al. and Huang and Adams [6, 25]. To simulate the *in vivo* dilution of saliva, 5 mL of a sterile electrolyte solution (6.2 g L^{-1} NaCl, 2.2 g L^{-1} KCl, 0.22 g L^{-1} $CaCl_2$, 1.2 g L^{-1} $NaHCO_3$, all purchased from Merck) was added to 22.5 mL of cell suspension. An aliquot was withdrawn (control G1) and then lysozyme (Sigma-Aldrich, Chemie Gmbh P.O. Steinheim, Germany) was added to give a final concentration of 0.01%. To simulate the gastric environment, 3 mL of electrolyte solution containing 0.3% pepsin (final concentration) (Sigma-Aldrich) at pH 5.0 was added to the cell suspension and an aliquot was taken without further incubation (sample G2). Then, the pH curve in the stomach was reproduced by adding 1 M HCl (Merck) to the cell suspension, at an initial pH of 5.0 which was then decreased to 4.1,

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3.0, 2.1 and 1.8. To mimic normal gastric emptying [6], aliquots of the suspension were collected after successive incubations of 20 min at 37 ºC at each pH (samples G3 to G7). To simulate the intestinal stress, samples 3, 4 and 5, were adjusted to pH 6.5 with 1 M $NaHCO_3$ (Merck), then mixed with 4 mL of a sterile electrolyte solution (5 g L^{-1} NaCl, 0.6 g L^{-1} KCl, 0.3 g L^{-1} $CaCl_2$, all purchased from Merck), containing 0.45% bile salts and 0.1% pancreatin (final concentrations, both from Sigma-Aldrich) at pH 8.0. After 120 min of incubation at 37 ºC, simulating the conditions of the duodenum, fractions of suspensions were collected (samples GI3, GI4, GI5). To avoid interference of milk proteins in the fluorescence determinations of cell viability, all samples (gastric or gastrointestinal) were treated as follows. First, pH of the sample was neutralized to 6.5 with 1 M NaOH (Merck). Then, 1% $C_6H_5Na_3O_7$ $2H_2O$ (Merck) was added to provoke casein micelle dispersion, as previously described [26]. The bacterial cells were then sedimented by centrifugation (10,000 × g for 10 min), washed twice by resuspension in 2.5 mL of PBS buffer pH 7.5 (10 mM Na_2HPO_4, 1mM KH_2PO_4, 140 mM NaCl, 3mM KCl, all purchased from Merck) and sedimented as described above. Finally, cells were resuspended in 2.5 mL of PBS buffer pH 7.5 and analyzed for cell survival as detailed below.

Cell survival analysis

Untreated and treated suspensions were analyzed for growth on solid media by plating and for viability by use of fluorescent dye staining.

For measurement of colony forming units (cfu), samples were plated on the appropriate culture media as detailed above supplemented with 1.5% bacteriological agar (Scharlau, Barcelona, Spain) and colonies were counted after incubation for 48 h. Simultaneously, to test bacterial viability, samples were stained with the LIVE/DEAD® *Bac*Light™ bacterial viability kit (Molecular Probes, Inc.AA Leiden, The Netherlands)

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as described by Alakomi et al. [27]. This kit is based on a combination of two probes, SYTO9 and propidium iodide (PI). SYTO9 is a membrane-permeable nucleic acid stain (emission of green fluorescence), whereas PI enters only the cells with compromised membranes (emission of red fluorescence). Since PI has a higher affinity for DNA than SYTO9, it is able to displace it. Thus, viable cells are detected by green emission and damaged or dead cells by red emission.

To assses viability, the staining solution was prepared by diluting the commercial stock in 0.085% NaCl (Merck) to final concentrations of 0.167 mM SYTO9 and 1 mM PI. Then, 1 mL of each bacterial suspension in PBS (approximately 1×10^9 cfu mL^{-1} of *L. paracasei* and *B. lactis* or 3.5×10^8 cfu mL^{-1} of *L. acidophilus, L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) were mixed with 33 µL of the staining solution. The samples were incubated at room temperature in the dark for 15 min. Three aliquots of 200 µL of each mix were pipetted into three separate wells of a 96-well microplate. The green and red fluorescences of the bacterial suspensions were measured in a LS-50B automated fluorometer (Perkin-Elmer, Boston, MA, USA) by detection of emission of SYTO9 and PI at 530 nm and 620 nm, respectively, upon excitation at 488 nm and with slits of 5.0.

In order to calibrate LIVE/DEAD® *Bac*Light™ for viability assessments, standards were prepared by mixing non-viable cells with viable cells at 0, 20, 40, 60, 80 and 100%. Heat treatment (70ºC, 30 min) was used to prepare non-viable cells, whereas non-heat-treated cells from fresh culture were used as viable cells. The results showed that the Green/Red ratio of all the strains analyzed correlated linearly with the number of viable cells in the suspensions (R^2 = 0.97-0.99) (as an example the data obtained for *L. paracasei* is depicted in Fig. S1).

To automatically correct for possible pipetting errors, the ratio of green and red fluorescences obtained for control cells G1 was considered as 100% and the change in

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this ratio was used to calculate the viability in the G-stress samples G2-G7. Similarly, for the GI-stress experiments, the Green/Red ratios for samples G3, G4 and G5 were considered as 100% and changes in these ratios was used to calculate the viability in samples GI3, GI4 and GI5.

Confocal laser scanning microscopy

To confirm some results, treated and stained bacteria as describe above, were analyzed with a confocal laser scanning microscope (CLSM), Leica TCS-SP2-AOBS model (Leica Microsystems GmbH, Wetzlar, Germany). Confocal illumination was provided with a X63 magnification objective and numerical aperture of 1.4-0.60 and by Argon laser (488 nm laser excitation) with a long pass 520-565 nm filter (for green emission) and long pass 630-685 nm filter (for red emission). Image analysis was performed using FRET and FRAP software.

Caco-2 cell culture and adhesion assay

Caco-2 cells, originating from human colonic adenocarcinoma, were obtained from the human cell bank at the Centro de Investigaciones Biológicas (Madrid, Spain). These cells were used in their terminally differentiated state to mimic small intestine mature enterocytes. Caco-2 cells were grown in Men-Alpha Medium (Invitrogen, Barcelona, Spain) supplemented with 10% (v v^{-1}) heat-inactivated fetal bovine serum at 37 ºC in an atmosphere containing 5% CO_2. Intestine cells were seeded in 96-well tissue culture plates (Falcon Microtest ^{TM}, Becton Dickinson, Franklin Lakes, NJ, USA) at 1.25×10^4 cells per well and grown during 15 days to obtain a monolayer of differentiated and polarized cells. The culture medium was changed every 2 days. To study the adhesion of each strain, cells were overlaid with bacteria resuspended in DMEM medium (0.1 mL/well) and at a multiplicity of infection of 10 bacteria per

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epithelial cell. After a 1 h incubation period at 37 ºC under 5% CO_2 atmosphere, cells were washed three times with phosphate-buffered saline pH 7.1 (PBS), resuspended in 0.1 mL of PBS, and Caco-2 cells detached after addition of 40% glycerol and freezing at –70 ºC. To determine the number of cell-associated bacteria, appropriate dilutions were plated onto agar plates containing media specific for each strain. Each adhesion assay was conducted in triplicate.

Cytokine quantification: Enzyme-Linked Immunoabsorbent Assay (ELISA)

To analyze cytokine secretion, supernatants from bacteria-treated cells were collected after 8 and 24 h and frozen at –70 ºC until assayed. The concentration of the pro-inflammatory cytokines IL-6 and IL-8 respectively in the supernatants of 8 and 24 h (optimal conditions of detection) were determined using commercially available ELISA kits (Immuno tools GmbH, Heidelberg, Germany). Each assay was performed in triplicate.

Results

The tolerance to digestive tract stress of three probiotic bacteria and the two yoghurt strains that constitute the microbiota of a commercial synbiotic product (Synbiotic Drink) was investigated. Bacteria, included in skim milk acidified at pH 4.6, were incubated in conditions that simulated the major factors influencing the survival of the ingested micro-organisms during their passage through the gastrointestinal tract (Fig. 1). We have considered three relevant factors, the effect of lysozyme, the influence of acid pH values with pepsin (gastric stress, G-stress) and the further action of bile salts and pancreatin (intestinal stress, GI-stress), simulating successive gastric delivery of bacteria to the intestine during digestion [6]. These analyses were performed using

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bacteria suspended in acidified milk (pH 4.6) to evaluate the matrix effect on ingestion of bacteria in fermented milks. The methodology is shown schematically in Fig 1. The survival of each strain was analyzed by fluorescence measurements and plate counts at different time intervals. The effect of lysozyme alone had no pronounced effect on viability of any strain (data not shown). Fig. 2 illustrates the effect of the G-stresses at different pH values on the viability of the studied bacterial strains. Presentation of the data as percentages of the values obtained for untreated cultures revealed that the pattern of cell survival detected by fluorescence (Green/Red ratio) was similar to that observed by plate counting for most of the strains analysed. However, there were greater discrepancies between survival as measured by plate counting as opposed to fluorescence readings for *L. acidophilus*, *L. delbrueckii subsp. bulgaricus* and *S thermophilus*, when exposured to pH values below 4.1 (G5 through G7). The samples containing lysozyme and pepsin at pH 5.0 did not drastically affect the viability of any of the strains (sample G2 *versus* control sample G1). Under these conditions, no significant reduction of plate counts was observed for any of the bacteria, and only a 30% reduction of the Green/Red ratio was detected in the *S. thermophilus* suspension (Fig. 2). Exposure of cell suspensions to decreasing pHs (5.0, 4.1, and 3.0) and further incubation for 20 min at 37° C (samples G3, G4, and G5), caused a slight but progressive reduction of viability in the yoghurt strains (Fig. 2). After incubation at pH 3.0 (sample G5), the percentage of Green/Red ratio remained higher than 80% for *L. paracasei* and *L. acidophilus,* whereas it decreased to 55%, 66%, and 72% for *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus,* and *B. lactis* respectively. These decreases correlated approximately with reductions in plate counts to 17%, 33 % and 67% of the values of control untreated cells (Fig. 2). When the simulated G-stress was set at pH 2.1 (sample G6), all the strains except *L. acidophilus*, showed a sharp reduction of the Green/Red ratio (86-76% loss of viability) (Fig. 2). Similarly, the

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capability of all bacteria (except for *L. acidophilus*), to form colonies was reduced between 4 to 5-log-units after incubation of cell suspensions at pH 1.8, which simulated the last gastric emptying to the intestine, and the Green/Red ratio was reduced by 82% to 86%. By contrast, after G-stress at pH 1.8, *L. acidophilus* still showed 53% viability as estimated by fluorescence and only approximately 1-log-unit reduction in plate counts. The above results indicate that for most of the analyzed bacteria, gastric emptying at pH values below 3.0 should deliver low doses of viable cells into the intestine.

Therefore, to evaluate the transit tolerance of strains in conditions simulating the duodenal, aliquots taken from G3, G4 and G5 where further incubated with 0.45% bile salts and 0.1% pancreatin. Table 1 and Table S1 show comparative results of resistance to G and GI-stress. In general, the reduction of plate counts caused by duodenal secretions could be correlated with the degree of severity of the previous gastric incubation of cell suspensions. The highest sensitivity to the simulated intestinal stress was found for *B. lactis*, with a drastic reduction of survival (4.4 log units decrease, Table 1) even after G-stress at pH 5.0. This loss of capability to form colonies was also associated with reduction of the Green/Red ratio to 12% of those of the delivered cells (Table S1). By contrast, *L. acidophilus*, with a cell survival of 64% after gastric treatment at pH 3.0, as measured by plate colony counts, was not further affected by intestinal stress (Tables 1 and S1). These results correlated with its high survival rate observed at low pH values (Fig. 2). However, this was not in strict agreement with the Green/Red ratio, which decreased from 88% (G5) to 18% (GI5) under these gastrointestinal stress conditions (Table S1). Surprisingly, *S. thermophilus* showed a good rate of survival to intestinal stress even after previous gastric exposure to pH 3.0 with a reduction of 2.4 log-units (Table 1), corresponding to a 3% growth ability and a 23% viability of the cells surviving G-stress (Tables 1 and S1).

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As shown in Figure 3, the adhesion of strains ranged from 1% to 9%, with *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* (7%) showing the highest levels of adherence, whereas *B. lactis* and *L paracasei* showed the lowest capability (2%). The ability of the five bacterial strains to interact with the enterocytes prompted us to determine their influence on the secretion of pro-inflammatory cytokines by Caco-2 cells. Caco-2 cells constitutively secreted detectable levels of both IL-6 and IL-8 (Table 2), but these were not modulated by any of the strains tested (Table 2). Therefore the adherence of the bacterial cells does not appear to modify the basal state of activation of the epithelial cell line. Nevertheless, it should be borne in mind that this lack of stimulation of cytokine expression *in vitro* may not be indicative of what occurs in the *in vivo* situation.

Discussion

Fermented milks are a widely used vehicle for delivering probiotic bacteria in food. Strains of *L. acidophilus*, *L. casei* group and *B. lactis* predominate in commercial probiotic products [28, 29]. In this work, we have analyzed three strains of the aforementioned species and two yogurt strains for their sensitivity to digestive tract conditions, and their ability to interact with human intestinal cells.

To assess the resistance of these strains to GI-stress, we have studied their *in vitro* survival under conditions that simulated the normal physiological conditions of the GI tract, such as the presence of lysozyme and pepsin, sequential gastric emptyings at increasingly lower pH values, and the presence of bile salts and pancreatin. In addition, we have analyzed the applicability of a microplate fluorochrome assay as a rapid assessment of bacterial viability. This test has been previously used for analysis of bacterial viability in probiotic preparations and dairy products [14, 30, 27, 15] as well as

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for testing gastrointestinal tolerance of *Bifidobacterium* [31, 8]. However, to our knowledge, this is the first report on the general application of this fluorescence system for the assessment of viability of probiotic strains belonging to different genera after gastrointestinal stress.

Strain survival estimated by fluorescence or by plate counts behaved generally in a similar manner after G-stress (Fig. 2). However, after GI-stress two different behaviours were observed. In the case of *L. acidophilus,* a higher cell recovery was detected by plate counts than by fluorescent measurements whereas, for the other four bacterial strains analyzed, the cell survival detected by fluorescent measurements was higher than that observed by plate count (Tables 1 and S1). A survival rate that is higher when measured by Green/Red fluorescence ratio, than when measured by plate colony counting is normally indicative that a proportion of the cells are still viable but not readily cultivable (e.g. *L. rhamnosus* strains subjected to acid and bile salt stress only yielded countable colonies after 168 h of incubation [32]) An addition explication would be that the GI-stress conditions provoke the formation of chains of cells or cell clumping; each chain or clump would only give rise to single colony on plate counts but would still be correctly enumerated by fluorescence. The possibility that high fluorescence measurements could be due to non-specific staining of residual caseins present in the samples was investigated by confocal microscopy which showed that for all strains tested only the bacteria were stained by SYTO9 or propidum iodide (Fig. 4). As expected, green (viable) cells were predominant in untreated cultures, whereas red (non-viable) cells were in the majority in suspensions subjected to gastrointestinal stress. The situation of *L. acidophilus*, where the colony counts were substantially higher than the fluorescence measurements, is harder to explain, though it is possible that GI-stress causes cell surface / cell wall changes in this organism that radically alter the penetrability of the fluorescent stains.

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Recent reports give evidence of the survival of yogurt bacteria in the upper compartments of human digestive tract [33; 34]. Our study revealed the significant impact of the pH on the survival of the strains analyzed. The results showed that most of the strains did not tolerate pH values below 3 (Fig. 2); but that above this value significant numbers of viable bacteria reach the intestine from the gastric content.

It has been proposed that damage of bacterial cell envelope by low pH could make the cells more susceptible to bile action on cell membranes [35]. The analysis of GI-stress performed here showed that indeed bacterial suspensions sublethally damaged by acid stress at pH 3, were more sensitive to intestinal secretions than those exposed to pH 5 (Table 1) Also, cell suspensions treated at pH values below 3 were highly susceptible to intestinal secretions, yielding counts close to zero (results not shown) and fluorescence measurements below the detection limits. This contrasts with the findings of Noriega et al. [36] who suggested that in *Bifidobacterium* a previous exposure to low pH in the stomach, might cause a transitory rise of bile resistance, thus increasing its survival in the duodenum. This kind of adaptation has recently been demonstrated to be due to an increase of the intracellular ATP reserve [37]. Mättö et al. [38] have described that *B. lactis* Bb-12 also survived through the human gastrointestinal tract, since the bacterium was detected in the faeces of 79% of subjects consuming probiotic yoghurt. In this study *B. lactis* Bb-12 showed a drastic reduction of its ability to form colonies in samples at pH 5.0 (Table 1). However, its viability (around 10% of the untreated control as estimated by fluorescent measurements) remained constant even in samples treated at lower pH values, supporting resistance of the strain to bile stress. It has been reported that in some cases the possession of bile or acid resistance alters the ability of the strains to adhere to human mucus [39; 40]. Our analysis of adherence to Caco-2 cells showed that *B. lactis* Bb-12, along with the other strains analyzed, is able to interact with epithelial cells. Their detected adherence (2%-10%) was within the lower range of the

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values observed for mucus adhesion of other *Bifidobacterium* strains from faecal human origin [40].

The potential immuno-stimulating properties of bifidobacteria and LAB have attracted attention in recent years [41, 42]. IL-6 and Il-8 are multifunctional cytokines that play a major role in the acute-phase response to inflammatory stimulus [23]. Morita et al. [21] investigated 28 *Bifidobacterium* and lactobacilli strains for their ability to stimulate cytokine IL-6 and IL-8 production. Some of the *Bifidobacterium* strains induced secretion of IL-8, but this was not associated with the strains' binding affinity to Caco-2 cells. In this study, we have detected that the five strains studied have different adherence to Caco-2 cells (Fig. 3). However, none of the strains, including *B. lactis* Bb-12, exhibited the undesired property of inducing either IL-6 or IL-8 (Table 2).

The combination of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* is widely used as a starter for the production of yoghurt. *L. delbrueckii* subsp. *bulgaricus* LBY-27 and .S. *thermophilus* STY-31 strains showed relatively high levels of adhesion to Caco-2 cells, whereas *L. paracasei* susbp. *paracasei* LC-01 displayed a lower adhesion. (Fig. 3). Values approximately ten fold higher have been observed for *L. paracasei* susbp. *paracasei* strains ACA-DC221,333 and 3335 [43].

Of all the strains analyzed, the highest tolerance to GI-stress was observed in *L. acidophilus*. A high tolerance to acid pH has been frequently observed in different *L. acidophilus* strains [44], but this does not seem to induce significant bile salt, heat, or ethanol tolerance in the strains [45]. Recently, *L. acidophilus* bile tolerance has been linked to the expression of a bile-inducible operon encoding a two component regulatory system [46], and the results obtained in this work show that *L. acidophilus* LA-05 also possesses a high resistance to intestinal stress (Table 1). A recent study demonstrated that cell surface proteins of *L. acidophilus* NCFM can contribute to this organism's ability to attach to intestinal cells *in vitro* [47], and our results show that *L.*

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acidophilus LA-5 has a significant level of adhesion capability. We have also observed by microscopy (results not shown) that *L. acidophilus* LA-5 can form aggregates, which may be related to its adhesion to Caco-2 cells since, for the *L. acidophilus* M92 strain, a relationship has been shown between autoaggregation and adhesiveness, both mediated by proteinaceous components of the cell surface [48].

Conclusions. The overall results indicate that most of gastric emptying would release a large number of viable probiotics and yogurt strain cells into the intestinal tract, and demonstrate differences between bacterial species with respect to their sensitivity to gastric and intestinal secretions. It also indicates that the *in vitro* model and the fluorescent detection used to evaluate the gastrointestinal stress responses of probiotic and yogurt bacteria is a reliable tool for high throughput screening of bacterial survival. Moreover, the high levels of adhesion to epithelial cells observed for *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* highlight the interest to elucidate the molecular mechanisms of these interactions. Finally, the absence of induction of the cytokines IL-6 and Il-8 in human intestinal epithelial cells, indicates that all the bacterial strains tested in this study can attach to the epithelial cells without triggering local inflammatory response.

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Legends to the figures

Figure 1. Schematic representation of the *in vitro* digestive tract model. Aliquots of reconstituted milk at pH 4.6 were individually inoculated with cellular pellets of each of the indicated strains after growth in their specific media and conditions as described in Materials and Methods. Subsequently, the cells were subjected to G- and/or GI-stress at 37º C. To simulate G-stress, cells were exposed to pH 5.0 at initiation (sample G2) and to sequential incubations at 37 ºC for 20 min at each pH 5.0, 4.1, 3.0, 2.1 and 1.8 (samples G3, G4, G5, G6 and G7, respectively). To simulate GI-stress, cells were subjected to G-stress as described above (conditions 2, 3, 4 and 5) and then, samples were adjusted to pH 6.5 and treated with 0.45% porcine bile salts and 0.1% pancreatin (samples GI3, GI4 and GI5).

Figure 2. Analysis of cell survival after gastric stress. The indicated bacterial strains were subjected to various G-stresses (G2, G3, G4, G5, G6 or G7) as described in Fig. 1 and in Materials and Methods. Cell viability was analysed by fluorescence (black bars) and by plate counting (white bars).The values are the mean of three independent experiments, and are expressed as a percentage of the values for untreated control samples. 100% control values for Green/Red fluorescence ratio for *L. paracasei* subsp. *paracasei*, *L. acidophilus, B. lactis*, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were respectively 8.65, 9.87, 10.80, 9.88 and 9.04. 100% control values for plate counting for *L. paracasei* subsp. *paracasei*, *L. acidophilus, B. lactis*, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were respectively $1.1x10^9$, $6.0x10^7$, $1.4x10^9$, $3.0x10^8$ and $4.0x10^8$ cfu mL^{-1}

Figure 3. Adhesion of bacterial strains to Caco-2 cells after infection with 10 bacteria per epithelial cell, followed by 1 h incubation at 37 ºC in an atmosphere containing 5%

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CO_2. Adhesion levels are expressed as the % of cfus adhered after three washes with PBS at pH 7.1. Each adhesion assay was conducted in triplicate. Vertical bars represent the standard deviations of three independent assays.

Figure 4. Detection of stained cells by confocal microscopy. Cell-milk suspensions of the indicated bacteria untreated (left panel) or subjected to gastric treatment at pH 5.0 and further intestinal stress (right panel) were stained and analyzed under confocal microscope as described in Materials and Methods for visualization of viable (green) and non-viable (red). Bar = 20 μm

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Table 1. Comparative bacterial survival to gastric (G) and gastrointestinal (GI) stresses as detected by fluorescent staining and plate counting.

Bacteria	pH 5.0				pH 4.1				pH 3.0			
	Cell counts (log cfu mL^{-1})		Fluorescence (Green/Red)		Cell counts (log cfu mL^{-1})		Fluorescence (Green/Red)		Cell counts (log cfu mL^{-1})		Fluorescence (Green/Red)	
	G3-stress*	GI3-stress*	G3-stress	GI3-stress	G4-stress	GI4-stress	G-4stress	GI4-stress	G5-stress	GI5-stress	G5-stress	GI5-stress
L. paracasei	9.06 (0.03)	5.77 (0.67)	6.69 (0.51)	1.97 (0.16)	9.07 (0.02)	5.62 (0.39)	7.85 (1.32)	1.77 (0.39)	9.02 (0.02)	2.37 (0.35)	8.30 (0.90)	1.07 (0.57)
L. acidophilus	7.58 (0.01)	7.56 (0.01)	8.72 (1.73)	1.66 (0.47)	7.54 (0.13)	7.37 (0.33)	8.12 (1.15)	1.54 (0.11)	7.31 (0.29)	7.33 (0.21)	8.74 (0.18)	1.61 (0.10)
B. lactis	9.14 (0.11)	4.75 (0.14)	9.15 (0.73)	1.10 (0.17)	9.12 (0.09)	4.52 (0.16)	8.92 (2.21)	1.09 (0.03)	9.02 (0.05)	3.83 (0.08)	7.80 (1.84)	0.88 (0.10)
L. delbrueckii	8.29 (0.39)	5.44 (0.42)	7.88 (1.21)	1.80 (0.16)	8.29 (0.24)	5.79 (0.30)	7.45 (0.32)	1.66 (0.12)	8.11 (0.20)	2.93 (0.25)	6.53 (3.06)	1.55 (0.17)
S. thermophilus	8.52 (0.01)	7.43 (0.09)	5.43 (0.18)	1.08 (0.17)	8.07 (0.01)	6.41 (0.16)	4.83 (0.62)	1.10 (0.20)	8.78 (0.12)	6.38 (0.51)	5.01 (1.4)	1.14 (0.20)

*Bacteria were analyzed after incubation for 20 min at pHs 5.0, 4.1 and 3.0 (G-stress) and after a further incubation with 0.45% bile salts and 0.1% pancreatin (GI-stress; see materials and methods and Fig. 1). The results are the mean of three independent experiments (SD in parenthesis). The plate counts obtained for untreated cultures of *L. paracasei* subps. *paracasei*, *L. acidophilus, B. lactis*, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were respectively 9.03, 7.78, 9.14, 8.47 and 8.60 log cfu mL^{-1} . The values of Green /Red fluorescence ratio obtained for untreated cultures for *L. paracasei* subps. *paracasei*, *L. acidophilus, B. lactis*, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were respectively 8.65, 9.87, 10.80, 9.88 and 9.04.

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Table 2. IL-6 and IL-8 secretion by Caco-2 cells after exposure to bacterial strains.

Bacteria	IL-8 (pg mL^{-1}) 8h	Il-6 (pg mL^{-1}) 24h
L. paracasei	34.91 ± 1.06	0.12 ± 0.07
L. acidophilus	38.13 ± 3.57	0.03 ± 0.02
B. lactis	35.94 ± 12.51	0.04 ± 0.01
L. delbrueckii	25.21 ± 9.25	0.06 ± 0.02
S. thermophilus	25.23 ± 2.87	0.05 ± 0.01
Caco-2 cells	41.11 ± 21.04	0.09 ± 0.01

Cytokine production was assayed by ELISA titration on supernatants from differentiated Caco-2 cells after incubation with the indicated bacterial strains for 8 and 24 h at 37ºC in an atmosphere containing 5% CO_2. Values indicate the mean ± standard deviation of three independent experiments measured in triplicate.

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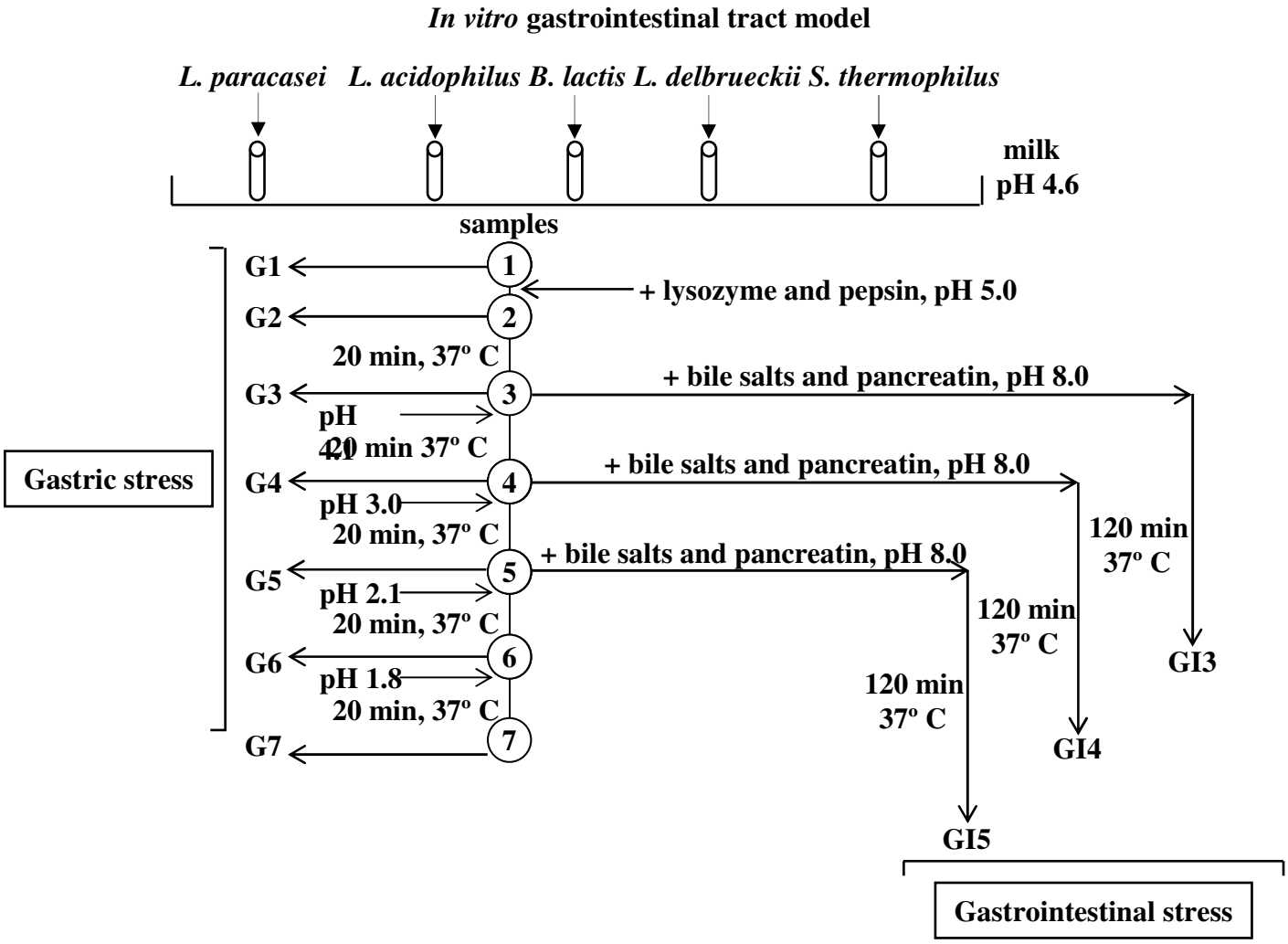
In vitro gastrointestinal tract model
L. paracasei *L. acidophilus* *B. lactis* *L. delbrueckii* *S. thermophilus*
milk
pH 4.6
samples
G1
1
+ lysozyme and pepsin, pH 5.0
G2
2
20 min, 37º C
+ bile salts and pancreatin, pH 8.0
G3
3
pH
20 min 37º C
Gastric stress
G4
4
+ bile salts and pancreatin, pH 8.0
pH 3.0
20 min, 37º C
120 min
37º C
+ bile salts and pancreatin, pH 8.0
G5
5
pH 2.1
20 min, 37º C
120 min
37º C
G6
6
pH 1.8
20 min, 37º C
GI3
120 min
37º C
G7
7
GI4
GI5
Gastrointestinal stress

Figure 1

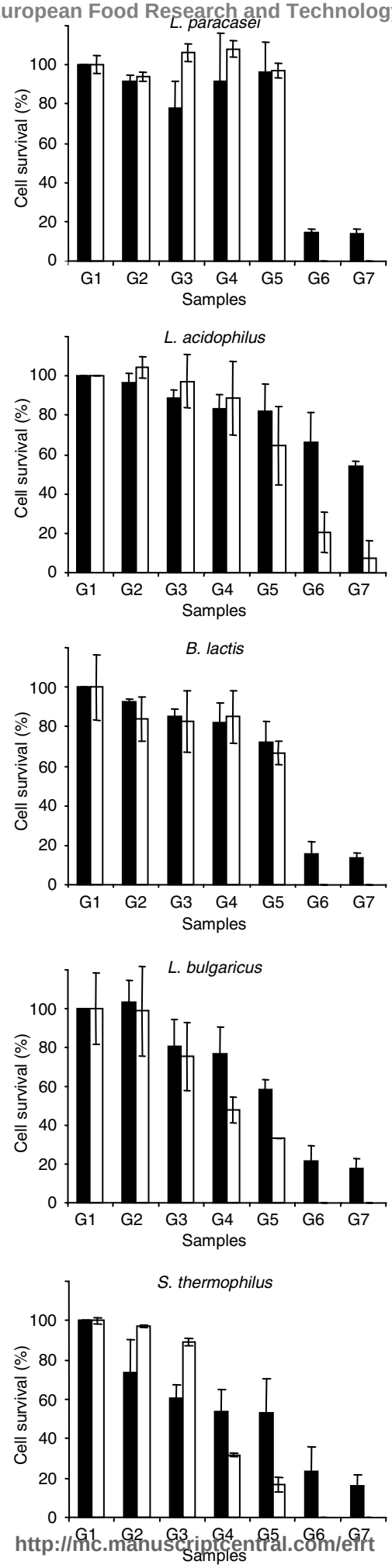
L. paracasei
L. acidophilus
B. lactis
L. bulgaricus
S. thermophilus
Cell survival (%)
0
20
40
60
80
100
G1
G2
G3
G4
G5
G6
G7
Samples

Figure 2

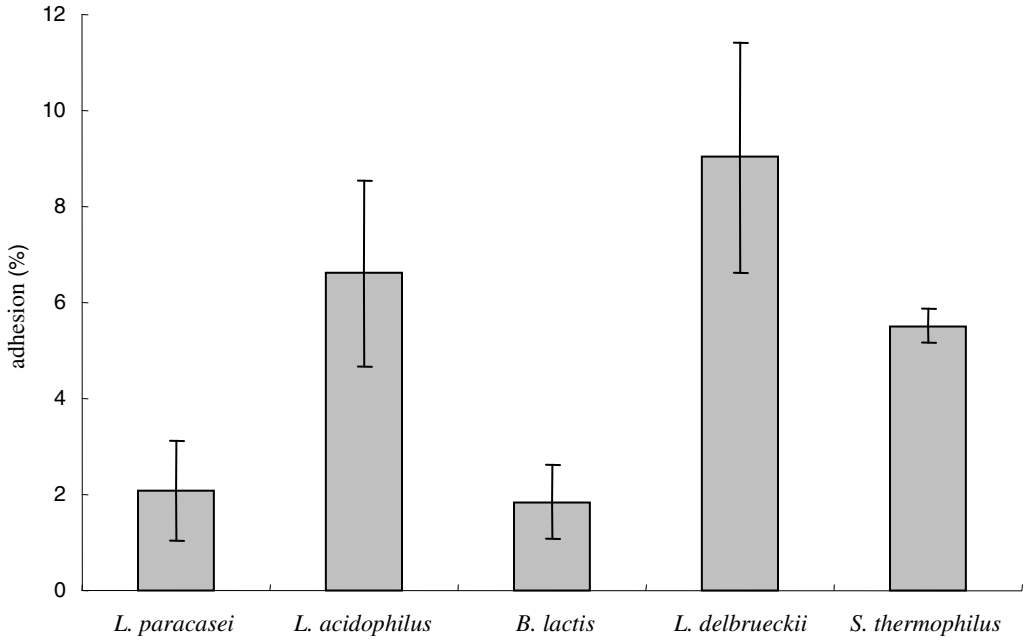
12
10
8
6
4
2
0
adhesion (%)
L. paracasei
L. acidophilus
B. lactis
L. delbrueckii
S. thermophilus

Figure 3

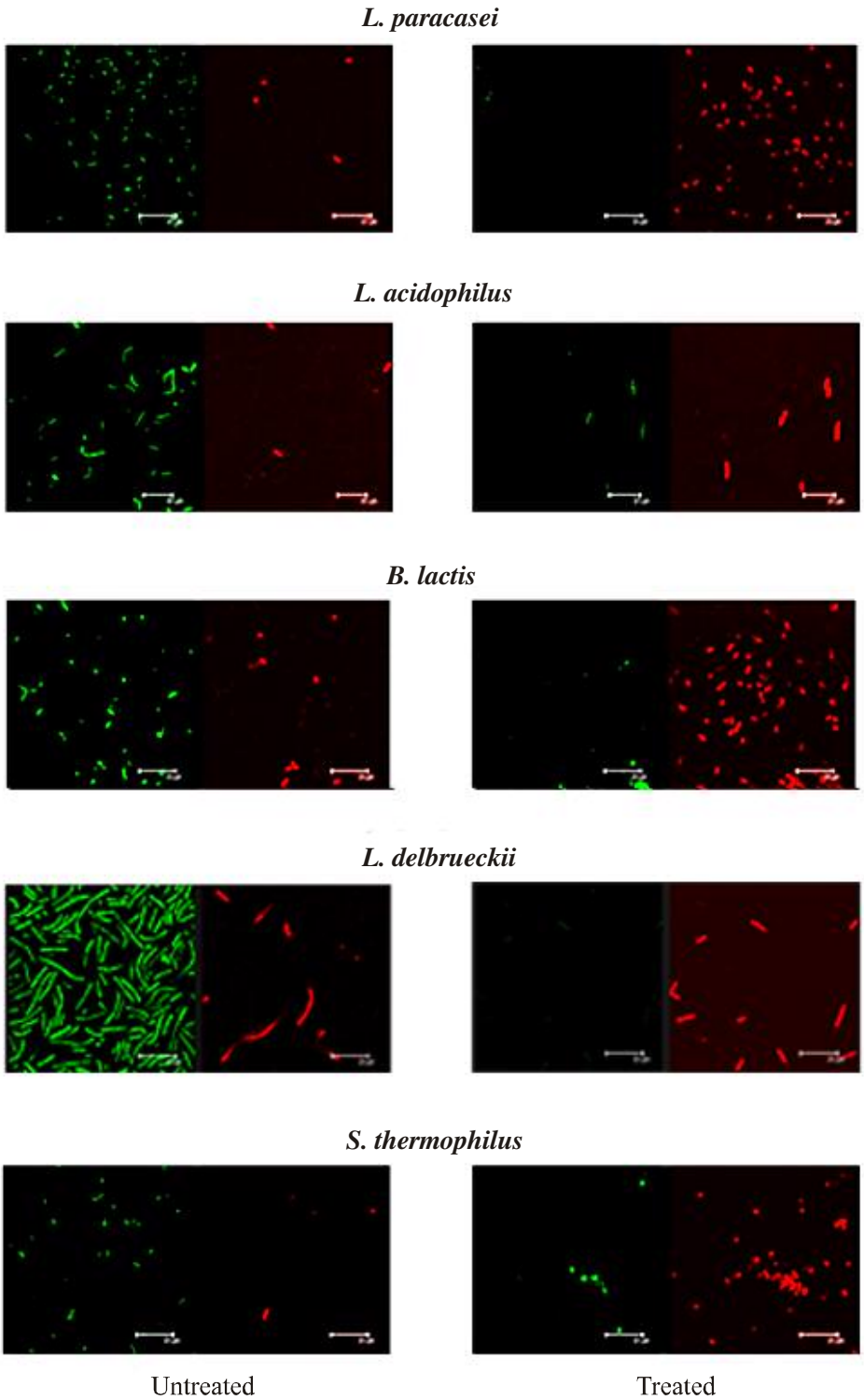
L. paracasei
L. acidophilus
B. lactis
L. delbrueckii
S. thermophilus
Untreated
Treated

Figure 4

Table S1. Comparative detection of bacterial resistance to gastric (G-stress) and gastrointestinal (GI-stress) by fluorescent staining and plate counting.

Bacteria	pH 5.0				pH 4.1				pH 3.0			
	Cell counts (% cfu mL^{-1})		Fluorescence (% Green/Red)		Cell counts (% cfu mL^{-1})		Fluorescence (%Green/Red)		Cell counts (% cfu mL^{-1})		Fluorescence (% Green/Red)	
	G3-stress*	GI3-stress*	G3-stress	GI3-stress	G4-stress	GI4-stress	G4-stress	GI4-stress	G5-stress	GI5-stress	G5-stress	GI5-stress
L. paracasei	100.33	0.13	77.34	29.44	108.04	0.06	90.75	22.54	97.24	<0.01	95.95	12.89
L. acidophilus	97.42	96.50	84.34	19.03	88.51	91.85	82.26	18.96	64.49	114	88.55	18.42
B. lactis	100.00	<0.01	84.72	12.02	84.96	<0.01	82.59	12.21	66.66	<0.01	72.22	11.28
L. delbrueckii	97.87	0.31	79.75	22.84	47.65	0.32	75.4	22.28	33.32	<0.01	66.09	23.73
S. thermophilus	99.06	6.99	60.06	19.88	31.84	2.67	53.4	22.77	16.90	2.66	55.42	22.75

*Bacteria were analyzed after incubation for 20 min at pHs 5.0, 4.1 and 3.0 (G-stress) and after a further incubation with 0.45% bile salts and 0.1% pancreatin (GI-stress; see materials and methods). The values of cfu mL^{-1} obtained for untreated cultures of *L. paracasei* subps. *paracasei*, *L. acidophilus, B. lactis*, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were respectively $1.1x10^9$, $6.0x10^7$, $1.4x10^9$, $3.0x10^8$ and $4.0x10^8$. The values of Green /Red fluorescence ratio obtained for untreated cultures for *L. paracasei* subps. *paracasei*, *L. acidophilus, B. lactis*, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were respectively 8.65, 9.87, 10.80, 9.88 and 9.04. In this table the results are represented as % of survival. For estimation of G-stress, percentages were calculated using as 100% initial values before treatment. For estimation of GI-stress, percentages were calculated using as 100% the corresponding values obtained with cells subjected to G-stress.

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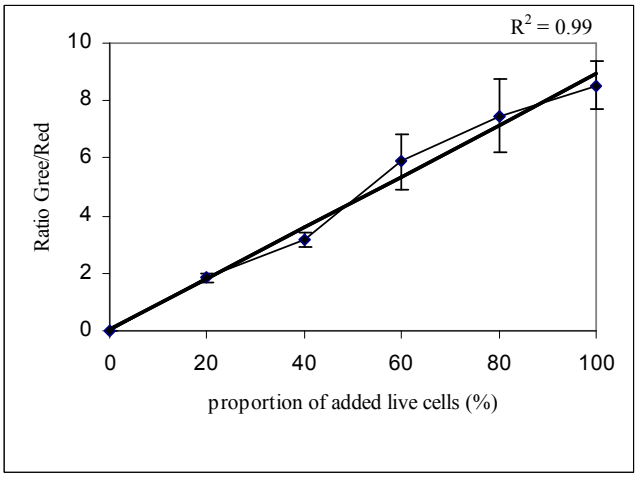

Fig S1. Standard curve for *L. paracasei*. Relationship between proportion of live bacteria (prepared by mixing live and heat-killed bacterial suspensions) and Green/Red fluorescence ratio (Ratio Green/Red).