Lactobacillus plantarum LB95 impairs the virulence potential of Gram-positive and Gram- negative food-borne pathogens in HT-29 and Vero cell cultures

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Listeria monocytogenes, Salmonella enterica and verocytotoxigenic *Escherichia coli* (VTEC) are amongst the most important agents responsible for food outbreaks occurring worldwide. In this work, two *Lactobacillus* spp. strains (LABs), *Lactobacillus plantarum* (LB95) and *Lactobacillus paraplantarum* (LB13), previously isolated from spontaneously fermenting olive brines, and two reference probiotic strains, *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* GG, were investigated for their ability to attenuate the virulence of the aforementioned pathogens using animal cell culture assays. In competitive exclusion assays, the relative percentages of adhesion and invasion of *S. enterica* subsp. *enterica* serovar Enteritidis were significantly reduced when the human HT-29 cell line was previously exposed to LB95. The relative percentage of invasion by *Listeria monocytogenes* was significantly reduced when HT-29 cells were previously exposed to LB95. In the cytotoxicity assays, the cell-free supernatant of the co-culture (CFSC) of VTEC with LB95 accounted for the lowest value obtained amongst the co-cultures of VTEC with LABs, and was significantly lower than the value obtained with the co-culture of VTEC with the two probiotic reference strains. The cytotoxicity of CFSC of VTEC with both LB95 and LB13 exhibited values not significantly different from the cell-free supernatant of the non- pathogenic *E. coli* B strain. Our results suggested that LB95 may be able to attenuate the virulence of Gram-positive and Gram-negative food-borne pathogens; together with other reported features of these strains, our data reveal their possible use in probiotic foods due to their interesting potential in preventing enteric infections in humans.

INTRODUCTION

Verocytotoxigenic *Escherichia coli* (VTEC), *Salmonella enterica* and *Listeria monocytogenes* are amongst the most important zoonotic agents worldwide. In 2013, the number of confirmed VTEC infections in humans increased and a total of 5196 food-borne outbreaks, including water-borne outbreaks, were reported in the European Union (EU). Most such cases were caused by the serogroup O157 (EFSA & ECDC, 2015). In spite of the decreasing trend of human salmonellosis cases in recent years in the EU, *Salmonella* is still the most frequent cause of reported food-borne outbreaks, with 82 694 cases confirmed in 2013. As in previous years, the two most commonly reported *Salmonella* serovars in 2013 were *S. enterica* subsp. *enterica* serovar Enteritidis and *S. enterica* subsp. *enterica* serovar Typhimurium, accounting for 39.5 and 20.2 %, respectively, of all reported serovars in confirmed human cases (EFSA & ECDC, 2015). In the USA, *Listeria monocytogenes*, *Salmonella* and *E. coli* O157 are the leading causes of death from food-borne infections, representing 75 % of reported cases (CDC, 2015).

Human listeriosis showed an increasing trend in the EU in the 2009–2013. In 2013, seven strong-evidence food-borne outbreaks caused by *Listeria monocytogenes* were reported by five EU Member States. These outbreaks resulted in 51 cases, 11 hospitalizations and three deaths, i.e. 37.5 % of all deaths due to strong-evidence food-borne outbreaks reported in 2013 (EFSA & ECDC, 2015).

Facultative intracellular food-borne pathogenic bacteria, e.g. Gram-negative *Salmonella* and Gram-positive *Listeria monocytogenes*, invade host cells owing to their specialized mechanisms enabling evasion of the phagocytic vacuole. *Salmonella* was the first bacterium shown to be able to use several invasion pathways, mediating either Trigger or Zipper entry processes (Boumart *et al.*, 2014). *Listeria monocytogenes* can induce its own internalization in various types of cells that are not normally phagocytic (Cabrita *et al.*, 2014; Cossart & Lebreton, 2014).

Extracellular bacterial pathogens, such as the Gram-negative *E. coli* O157 : H7, do not invade cells but adhere to epithelial surfaces and cause disease by secreting toxins. *E. coli* O157 : H7 colonizes the large intestine and secretes Shiga toxins, Stx1 and Stx2, as major virulence factors causing attaching and effacing intestinal lesions (Kalita *et al.*, 2014).

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.*, 2014). Probiotics include multiple bacterial species, from such distinct genera as *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, which have been found to generally promote and maintain a balanced intestinal microenvironment by preventing active viral and bacterial infections (Scott *et al.*, 2015). The competitive exclusion of potential pathogens, or the production of useful metabolites or enzymes, are widespread mechanisms commonly accepted to exist amongst most probiotics studied (Reid *et al.*, 2011; Kumar *et al.*, 2013). These reasons, along with the increasing awareness of their health-promoting effects, explain the increase in the consumption of functional products containing probiotic micro-organisms over recent decades. This has prompted an interest in the development of novel functional foods, or food formulations, as sources or carriers of probiotic strains (Peres *et al.*, 2012; Martins *et al.*, 2013).

The aim of this work was to investigate the role of two selected *Lactobacillus* spp. strains (LABs), isolated from spontaneously fermenting olive brines (Peres *et al.*, 2014), upon attenuation of the virulence potential of four food- borne pathogenic bacterial strains (*S.* Enteritidis, *S.* Typhi-

murium, *Listeria monocytogenes* and *E. coli* O157 : H7). Toward this main goal, the competitive exclusion proper- ties of the LABs against *Salmonella* and *Listeria monocyto- genes* in the human intestinal HT-29 cell line, as well as their effect on the attenuation of the cytotoxicity of *E. coli* O157 : H7 in Vero cells, were duly investigated.

METHODS

Bacterial strains. The two probiotics used as reference LABs were *Lactobacillus casei* Shirota (ACA-DC 6002) and *Lactobacillus rhamnosus* GG, provided by the Laboratory of Microbiology and Bio- technology of Food at the Agricultural University of Athens (Iera Odos, Greece). The two selected LABs used in this study, *Lactobacillus plantarum* (LB95) and *Lactobacillus paraplantarum* (LB13), came from industrial and homemade fermented olives of Portuguese cultivars (Peres *et al*., 2014), and were made available by Instituto de Tecnologia Química e Biológica, New University of Lisbon (Oeiras, Portugal). The pathogenic strains used were: *Listeria monocytogenes* EGDe, originally obtained from Institut National de la Recherche Agronomique (Tours, France), *E. coli* O157 : H7 (ATCC 43895), *E. coli* B from Coleção de Bactérias do Instituto Superior de Agro- nomia, University of Lisbon (Lisbon, Portugal), and *S*. Typhimurium (ATCC 14028) and *S*. Enteritidis from Instituto de Ciência Aplicada e Tecnologia, University of Lisbon (Lisbon, Portugal). Cultures were maintained on Tryptic Soy Broth (TSB; Difco) containing 15 % (v/v) glycerol (Merck) and stored at 280 ºC until use.

Bacterial culture media. All strains were grown at 37 ºC in the following media: *E. coli* strains in Brain Heart Infusion (BHI) broth (Oxoid), with shaking, or on BHI plates [BHI supplemented with 1.5 % (w/v) agar (Iberagar)], Tryptone Soy Yeast Extract Agar [TSA- YE with 1.5 % (w/v) agar], or Tryptone Bile X-Glucuronide (TBX) agar (Biokar). *Listeria monocytogenes* strains were grown on TSA-YE or PALCAM (Biokar). *Salmonella* strains were grown on TSA- YE or Xylose Lysine Deoxycholate (XLD) agar (Biokar). The LABs were grown in Man–Rogosa–Sharpe (MRS) broth (Biokar,) or onto MRS plates [MRS supplemented with 1.5 % (w/v) agar]; the strains were then grown under the conditions described for the virulence assays.

Cell lines and culture conditions. The human adenocarcinoma cell line HT-29 (ECACC 91072201) was used between passages 34 and 64, and the Vero cell line (ATCC CCL-81) was used between passages 68 and 71. Cells were routinely grown in 75 cm^2 flasks (Orange Scientifique) in RPMI 1640 medium (Sigma) with FBS (10 % (v/v) and L- glutamine (2 mM) (complete RPMI 1640 medium). Penicillin (100 $\mu g\ ml^{-1}$) and streptomycin (100 $\mu g\ ml^{-1}$) were always added to the culture medium, except to that used 24 h prior to the virulence assays. Cells were maintained in a humidified atmosphere using an incubator at 37 ºC under 5 % (v/v) CO_2 in air.

Adhesion and invasion assays. For adhesion and invasion assays, HT-29 cells were seeded in 24-well tissue culture plates for 3 days. Cell cultures were replenished with complete RPMI 1640 without antibiotics 24 h before the assays were performed. The adhesion and invasion of the bacteria to the epithelial cells were evaluated both separately and in competitive exclusion assays.

In all the assays, LABs (10^8 c.f.u. per well) and pathogenic strains (10^7 c.f.u. per well) were used at a multiplicity of exposure of 100 : 1 and 10 : 1, respectively.

Adhesion of strains

Fresh overnight culture of each strain was suspended in complete RPMI 1640 medium. In duplicated wells, HT-29 monolayers were inoculated with 300 µl suspension of a single strain per well. Plates were then incubated for 1 h at 37 ºC with 5 % (v/v) CO_2 in air. The non-attached bacteria were subsequently eliminated by washing the monolayer twice with 500 ml PBS. Cells with adherent bacteria were harvested with 500 µl active-trypsin-versene (ATV) (0.5 % trypsin/ 0.2 % ETDA) following incubation for 15 min. After appropriate decimal dilutions of the recovered suspensions, adherent LABs were enumerated on MRS, *Listeria monocytogenes* on PALCAM, *E. coli* B on TBX and *S. enterica* on XLD agar plates, and incubated for 24–48 h at 37 ºC. The adhesion ability of the strains was expressed as the number of adherent bacteria divided by the total number of bacteria added and then multiplied by 100.

For the exclusion assays, LABs were added to the wells with the HT-29 cell monolayers and incubated for 3 h. Non-attached bacteria were removed by washing with PBS as described above, and *L. mono- cytogenes* or *Salmonella* strains were then separately added and incubated for 1 h. The HT-29 monolayers were then washed with PBS as before, and adherent bacteria were released with ATV and plated as described above. The inhibition of adhesion of the pathogenic bacteria in the competitive exclusion assays with the LABs was expressed as a relative percentage of adhesion of the pathogen to HT-29 cells in the absence of previously incubation of HT-29 with LAB bacteria.

Invasion by pathogenic strains

Suspensions of pathogenic strains were added to HT-29 monolayers and incubated for 1.5 h at 37 ºC with 5 % (v/v) CO_2 in air. The monolayers were then washed twice with PBS. Complete RPMI 1640 medium containing 100 µg gentamicin ml^{-1} was added and incubated for 1 h. The medium was then removed, the monolayer washed with PBS and ATV added as described above. After appropriate dilutions of the recovered suspensions, bacteria were plated for enumeration of internalized bacteria as described above. The invasion ability of the strains was expressed as the number of invading bacteria divided by the total number of bacteria added and then multiplied by 100.

For the exclusion assays, LABs were added 3 h before the infections of HT-29 monolayers with the pathogenic strains as described above for the adhesion assay. Non-attached bacteria were subsequently removed by washing with PBS. Bacterial suspensions of pathogenic strains were then added to cell monolayers and incubated for 1.5 h at 37 ºC with 5 % (v/v) CO_2 in air. The monolayers were next washed twice with PBS, and complete RPMI 1640 medium containing 100 µg gentamicin ml^{-1} was added followed by 1 h incubation. The medium was then removed afterwards, the monolayer washed with PBS, ATV added and bacteria plated for enumeration of internalized bacteria as described above. The inhibition of the invasion of the pathogenic bacteria in competitive exclusion assays with the LABs was expressed as a relative percentage of the invasion of the pathogen in the absence of previous incubation of HT-29 with LAB bacteria.

Plaque-forming assay (PFA) without and with previous exposure to LABs. The procedures used to obtain confluent HT-29 cell monolayers and for PFA in 96-well tissue culture microplates were as described previously (Roche *et al.*, 2001; Neves *et al.*, 2008). Briefly, for PFA without previous exposure of the HT-29 to LABs, overnightgrown *Listeria monocytogenes* (TSA-YE, 37 ᵒC) was suspended in complete RPMI 1640 medium to a concentration of 4×10^8 c.f.u. ml^{-1} and aliquots of 25 μl serial dilutions of this suspension (10^2–10^7 *Listeria monocytogenes* c.f.u. per well) were used in duplicate to infect HT-29 cell monolayers. The microplates were subsequently incubated for 2 h at 37 ᵒC with 5 % (v/v) CO_2 in air. The suspensions were then removed and the monolayers washed twice with PBS. Cell monolayers were then incubated for 1.5 h with complete RPMI 1640 medium containing gentamicin (100 μg ml^{-1}). Afterwards, the medium was removed and replaced by complete RPMI medium supplemented with gentamicin (10 μg ml^{-1}) and 2.5 % (w/v) agarose. Once the agar medium had solidified, complete medium was added to the top of the agar medium to prevent cell starvation. Culture plates were incubated for 24–48 h at 37 ᵒC under 5 % (v/v) CO_2 in air. Using an inverted microscope, wells with appropriate numbers of plaques were counted 24 h after *Listeria monocytogenes* deposition on HT-29 cell mono- layers and counting was confirmed following 48 h of incubation.

For the PFA with previous exposure of HT-29 to LABs, each LAB cultured in MRS medium was harvested by centrifugation at 8123 g for 5 min. The bacterial pellet was then resuspended in 10 ml PBS and centrifuged as before. After removing the supernatant, the pellet was resuspended in complete RPMI 1640 medium at 4×10^9 c.f.u. ml^{-1} complete RPMI 1640 medium. Aliquots of 25 μl of each LAB suspension were added to each well of the P96 microplate containing the HT-29 monolayers. The microplates were incubated for 3 h at 37 ᵒC in 5 % (v/v) CO_2 in air. The medium was then removed and the monolayers were washed twice with PBS to remove non-attached LABs. The assay proceeded afterwards with the addition of *Listeria monocytogenes* to the corresponding wells, in duplicate, in order to reach bacterial cell concentrations of 10^2–10^7 c.f.u. per well. The microplates were subsequently incubated for 2 h at 37 ᵒC with 5 % (v/v) CO_2 in air and the assay proceeded as described above.

Cytotoxicity assay. The cytotoxic potential of an *E. coli* O157 : H7 strain was evaluated based on the method described by Maldonado *et al.* (2005), with some modifications. This cytotoxicity test was based on the release of lactate dehydrogenase (LDH) from Vero cells and was assessed using a Cytotoxicity Detection Kit^{PLUS} (Roche).

To determine the percentage cytotoxicity, A_{490} and A_{650} were measured and the differences in absorbance values (A_{490}–A_{650}) of each replica were calculated. The mean absorbance values were then calculated and the absorbance value of the background control (LDH contained in the assay medium) was subtracted from each. Low control (spontaneous LDH released) and high control (maximum LDH released from cells treated with lysis solution) were considered, according to Roberts *et al.* (2001), as:

$$\% \text{ Cytotoxitity} = [(A_{\text{exp}} - A_{\text{low control}})$$
$$/(A_{\text{high control}} - A_{\text{low concontrol}})] \times 100$$

where A denotes the absorbance.

Cell-free supernatant from pure cultures (CFS) and from co-cultures (CFSC)

The effect of different LABs on the attenuation of the cytotoxicity of *E. coli* O157 : H7 on Vero cell monolayers was tested using bacterial CFS of pure bacterial cultures or bacterial CFSC of *E. coli* O157 : H7 with LABs. In all the assays, *E. coli* O157 : H7 was used as positive control and non-pathogenic *E. coli* B was used as negative control.

For the assays with CFS, LABs and *E. coli* pure cultures were grown separately under the conditions described above. Aliquots (1.5 ml) from the pure cultures were centrifuged at 8160 g for 3 min. CFSs were then stored at 4 ℃.

For the assays with CFSC, pure cultures of each LAB and *E. coli* O157 : H7, grown as described before, were used to produce co-cultures of LABs with *E. coli* O157 : H7 in 20 ml BHI. For the negative control, in the co-culture with *E. coli* O157 : H7, the LAB was replaced by *E. coli* B. Incubation proceeded at 37 ℃ with shaking for 24 h. After this period, the concentration of each strain in the co-culture ranged from 1.32 to 7.75×10^8 c.f.u. ml^{-1} for LABs and from 2.43 to 9.75×10^8 c.f.u. ml^{-1} for the *E. coli* strains. Aliquots (1.5 ml) from each co-culture were centrifuged at 8160 g for 3 min. CFSCs were stored at 4 ℃.

Toxin preparation

Each bacterial cell pellet (see above) was resuspended in 75 µl polymyxin B sulfate solution (2 mg ml^{-1}) in order to release cell-bound toxins (Donohue-Rolfe & Keusch, 1983) and was incubated at 37 ℃ for 30 min in a shaker incubator. Samples were centrifuged at 8160 g for 5 min and the supernatant combined with the original CFS or CFSC. Toxin preparations were then filtered with 0.2 µm pore size syringe filters (Millipore) and the filtrates were either used immediately or kept at 4 ℃ for up to 24 h.

Cytotoxicity assay with toxins from CFSC and CFS

For the cytotoxicity assay, Vero cells were used to inoculate 96-well microplates (3×10^4 cells per well), and incubation proceeded for 24 h at 37 ℃ with 5 % (v/v) CO2 in air until a confluent monolayer was formed.

For the cytotoxicity assay with toxins from CFSC and after washing the cell monolayer twice with RPMI 1640 (serum-antibiotics-free), the Vero cell monolayers were exposed to 50 µl of each CFSC preparation and 50 µl of serum antibiotic-free RPMI 1640. For each experiment, the percentages of cytotoxicity values were not absolute as these were calculated based on the LDH

released by positive and negative controls. For the background and low control, 100 μl serum antibiotic-free RPMI 1640 was added. For the high control, 95 μl serum antibiotic-free RPMI 1640 was added. Each sample was tested in triplicate.

After 12 h of incubation at 37 ℃ in the presence of 5 % (v/v) CO2 in air, 5 μl lysis solution was added to the well for the high control and incubated at 37 ℃ for 15 min in an orbital incubator.

The release of endogenous LDH from the cytosol of damaged cells was measured by dispensing 100 μl freshly prepared reaction mixture (diaphorase NAD$^+$, iodotetrazolium chloride and sodium lactate) per well. The microplate was incubated in the dark for 5 min at room temperature as per the manufacturer's instructions. After incubation, 50 μl stop solution was added to the wells and shaken for 10 s. A_{490} and A_{650} were measured in a microplate reader (model 680; Bio- Rad), and the percentage cytotoxicity was determined as described above.

For the cytotoxicity assay with CFS, the Vero cell monolayers were likewise washed twice with RPMI 1640 medium (serum antibiotic- free) and exposed to 25 μl CFS from VTEC and 25 μl CFS from each LAB and 50 μl of serum antibiotic-free RPMI 1640. For the negative control, 25 μl CFS from VTEC was mixed with 25 μl CFS of *E. coli* B and 50 μl serum antibiotic-free RPMI 1640. The remainder of the procedure was the same as described for CFSC.

Expression of results and statistical analysis. All assays were performed with at least three independent trials. For the adhesion and invasion assays, the results were expressed as either an absolute or relative percentage. For PFA, the pathogenic potential of the isolates was expressed as the mean logarithm of the number of plaques formed (for 10^7 *Listeria* cells per well) (log PFA). For the cytotoxicity assays, the obtained differences of absorbance (A_{490}–A_{650}) were submitted to a rank transformation for normalization of the data. Agreement to a normal distribution of the data generated by the adhesion, invasion, PFA and cytotoxicity assays was checked using the Anderson–Darling test, and the homogeneity of the variance was confirmed by Levene's test. Comparisons between the percentages of adhesion, percentages of invasion and log PFA values were then performed via ANOVA using Tukey's multiple comparison tests. The comparisons between the rank transformation cytotoxicity values were performed using Schoeffer's comparison test. All statistical analyses were performed using Statistica version 7.0 (StatSoft).

RESULTS AND DISCUSSION

Adhesion and invasion ability of strains to HT-29 cells

The ability to adhere to or to colonize epithelial cells is essential (and thus a prerequisite trait) for probiotic bacteria (Hill *et al.*, 2014). By the same token, adhesion of pathogens to the intestinal epithelial surface is a key preliminary step in pathogenesis and is central to intestinal colonization (Dimitrov *et al.*, 2014).

The attachment profiles of *Lactobacillus plantarum* (LB95), *Lactobacillus paraplantarum* (LB13), the two probiotics *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* GG, and the enteropathogenic strains of *Salmonella* and *Listeria monocytogenes* to HT-29 cells were investigated. Attachment of the LABs varied from 0.7 to 1.8 %. *S.* Enteritidis attached to HT-29 cells at significantly higher numbers ($P < 0.05$) (17.8 %) than those of *S.* Typhimurium (6.4 %)

and *Listeria monocytogenes* (3.4 %) strains (Fig. 1a). Gagnon *et al.* (2013) showed that *Salmonella* adhesion to HT-29 cell depends on the serovar, with the higher relative abilities to adhere exhibited by *S.* Enteritidis. Our results confirm this finding and suggest that *S.* Enteritidis has a higher ability to adhere to the intestinal epithelium than *S.* Typhimurium.

However, there were no significant differences ($P>0.05$) amongst adhesion of LABs to HT-29 cells (Fig. 1a). Several studies showed that the percentage of LAB adhesion to gastric cells and Caco-2 cells is strain-dependent (Pisano *et al.*, 2014; Delgado *et al.*, 2015). However, Peres *et al.* (2014) showed that the adhesion of *Lactobacillus plantarum* (LB95) and *Lactobacillus paraplantarum* (LB13) strains to Caco-2 cells was not significantly different from that of *Lactobacillus casei* Shirota, which is consistent with our results pertaining to HT-29 cells.

When the percentages of invasion of the *S.* Typhimurium and *S.* Enteritidis strains were compared to each other, a significant difference ($P<0.05$) between the two strains was observed (Fig. 1b). *Listeria monocytogenes* and *S.* Typhi- murium were significantly more invasive ($P<0.05$) than *S.* Enteritidis. Interestingly, *S.* Enteritidis adhered to a greater extent, but invaded to a lesser extent the HT-29 epithelial cells than *S.* Typhimurium (Fig. 1a, b). Nevertheless, Gagnon *et al.* (2013) showed that the higher relative ability to invade was shown by *S.* Enteritidis. However, those authors found that *Salmonella* invasion using the HT-29 cell model is also dependent on the serovar, which seems to support the apparent contradiction associated with our results.

Competitive exclusion of *S. enterica* and *Listeria monocytogenes* by LABs

Anti-adhesiveness of the pathogens by probiotics has been explained by secretion of antimicrobial substances (e.g. organic acids, bacteriocin or hydrogen peroxide) by the probiotic strains, secretion of proteins able to degrade carbohydrate receptors, establishment of biofilms, and/or production of receptor analogues and biosurfactants (Vuotto *et al.*, 2014). The inhibition of the pathogens could also be related to the competition, by probiotics and pathogens, for specific receptors (Bienenstock *et al.*, 2013) or with such other factors such as co-aggregation of both strains (Vuotto *et al.*, 2014). In most cases, adhesion to mammalian cells, one of the most widely used tests, has been used for single strains (Papadimitriou *et al.*, 2015). In the present work, a competitive exclusion assay was used to investigate the effect of pre-exposure of HT- 29 cells to four LABs (LB95 and LB13 from fermented olives, and two probiotic reference strains) in terms of their impact upon adhesion and invasion ability of three pathogenic strains (*S.* Enteritidis, *S.* Typhimurium and *Listeria monocytogenes*). The results showed that the four LABs tested underwent a reduction in terms of initial adhesion and invasion of *S.* Typhimurium, *S.* Enteritidis and *Listeria monocytogenes* to <50 % (Fig. 2a, b). Wang *et al.* (2014) found that four *Lactobacillus plantarum* and *Lactobacillus casei* strains could strongly antagonize the adhesion and invasion of *Campylobacter jejuni* to HT-29 cells. Using Caco-2 cells, *Lactobacillus plantarum* was also shown to be the most effective in terms of its anti-adherence ability against *S.* Typhimurium and *Listeria monocytogenes* amongst three LABs studied

(Sribuathong *et al.*, 2014). With regard to adhesion and invasion inhibition of *S.* Typhimurium, no significant differences ($P>0.05$) were found amongst the four LABs (Fig. 2a, b). However, the relative percentages of adhesion and invasion for *S.* Enteritidis were significantly reduced ($P<0.05$) when the HT-29 cells were previously exposed to either *Lactobacillus plantarum* (LB95) or *Lactobacillus casei* Shirota when compared to *Lactobacillus rhamnosus* GG or *Lacto- bacillus paraplantarum* (LB13) (Fig. 2a, b). Similarly, previous exposure of HT-29 cells to LB95 resulted in a significantly lower ($P<0.05$) invasion of *Listeria monocytogenes* relative to previous exposure to the other LABs (Fig. 2b). In the PFA, the LABs significantly reduced ($P<0.05$) the virulence potential of *Listeria monocytogenes*, except for *Lactobacillus rhamnosus* GG, but no significant differences amongst LABs were detected (Fig. 3). According to Peres *et al.* (2014), the strain LB95 presents different probiotic features. Our results have extended these findings and shown that LB95 possesses a stronger antagonistic activity, against both *S.* Enteritidis and *Listeria monocytogenes* strains than the other LABs analysed, including the reference probiotics.

Cytotoxicity of *E. coli* O157 : H7

The putative protective effect of LAB supernatants against the cytotoxic potential of an *E. coli* O157 : H7 strain on Vero cells was analysed, including the use of bacterial CFS and CFSC. Recent works have investigated the effect of probiotics on pathogen-mediated cytotoxicity in mammalian cells using the CFS approach (Wong *et al.*, 2013; Bermu-dez-Brito *et al.*, 2014). As the impact of an individual pro- biotic is influenced by the composition of the microbiota (Howarth & Wang, 2013), the present investigation included both CFS and CFSC. However, our results (data not shown) demonstrated that CFSC-mediated cytotoxicity was not significantly different from CFS-mediated cytotoxicity, thus suggesting that the presence of the VTEC strain in the co- cultures did not hamper the performance of the LABs. The observed differences suggest that the co-culture of each LAB with the VTEC strain significantly reduced ($P<0.05$) VTEC cytotoxicity (5.9 –8.5 %) when compared with the positive control, i.e. VTEC (32 %), and with the negative control, i.e. non-pathogenic *E. coli* B (4.8 %) (Fig. 4). The CFSC cytotoxicity of VTEC grown with *Lactobacillus plantarum* (LB95) displayed the lowest value and was significantly lower ($P<0.05$) than the cytotoxicity (8.5 %) of VTEC in co-culture with each probiotic reference strain (*Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* GG). In addition, the CFSC cytotoxicity of VTEC when co-cultured with *Lactobacillus plantarum* (LB95) and *Lactobacillus paraplantarum* (LB13) presented a value (5.3 %) not significantly different (Pw0.05) from that displayed by the negative control (4.8 %) (Fig. 4). These results reinforce the importance of the composition of the microbiota upon the effect of probiotics, as claimed by Howarth & Wang (2013). However, the synergistic effect resulting from growing *Lactobacillus plantarum* (LB95) and *Lactobacillus paraplantarum* (LB13) together upon the cytotoxicity of VTEC has not been reported to date, and suggests that the presence of LB13 enhances attenuation of the cytotoxicity of VTEC toxins by LB95. Hence, complete remission of VTEC cytotoxicity could

be achieved by considering the joint use of *Lactobacillus plantarum* (LB95) and *Lactobacillus paraplantarum* (LB13).

In regard to *Salmonella* and *Listeria monocytogenes* virulence attenuation, *Lactobacillus plantarum* (LB95) provided the most effective protection against exposure to the VTEC toxins. Several authors have shown, through competition tests, that some *Lactobacillus plantarum* strains present good anti-adherence and anti-invasion abilities against *S.* Typhimurium and *Listeria monocytogenes* to intestinal cells (Botes *et al.*, 2008; Botta *et al.*, 2014; Sribuathong *et al.*, 2014). Moreover, the amount of valuable bacteriocins obtained from *Lactobacillus plantarum* species is worth mentioning and proves that it is one of the most important pro- biotic species amongst the LABs (Silva Sabo *et al.*, 2014).

To the best of our knowledge, this is the first report to demonstrate the abilities of LABs to inhibit the adhesion and invasion of Gram-positive and Gram-negative food- borne pathogens, using competitive exclusion tests against Typhimurium, *S.* Enteritidis and *Listeria monocytogenes*, and resorting to two cytotoxicity assays (with CFS and CFSC) against *E. coli* O157 : H7. In the five virulence/cytotoxicity tests, the LB95 strain presented better than or equal results to the probiotic strains *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* GG used as reference. Taken together, our findings suggest that *Lactobacillus plantarum* LB95, isolated from Portuguese spontaneous olive fermentation, should be considered as a potential probiotic strain with an interesting potential in prevention of enteric infections in humans. *In vivo* study models for pathogen infections are currently in progress to confirm the results obtained *in vitro* (P. Peres and others, personal communication), aiming at future development of novel health-promoting foods of plant origin.

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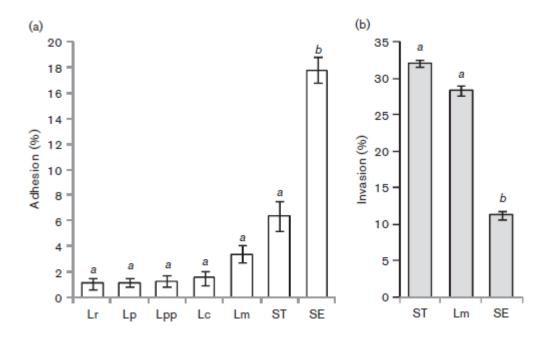
Fig. 1. (a) Bacterial adhesion to HT-29 cells and (b) enteropathogenic invasion of HT-29 cells. The percentages of adhesion and invasion were calculated relative to the numbers of c.f.u. initially added to the HT-29 cell monolayers. The data are mean±SD of three independent experiments, each performed in triplicate. Different letters above the columns indicate significant differences ($P < 0.05$) between values. Lr, *Lactobacillus rhamnosus*; Lp, *Lactobacillus plantarum*; Lpp, *Lactobacillus paraplantarum*; Lc, *Lactobacillus casei*; Lm, *Listeria monocytogenes*; ST, *S.* Typhimurium; SE, *S.* Enteritidis.
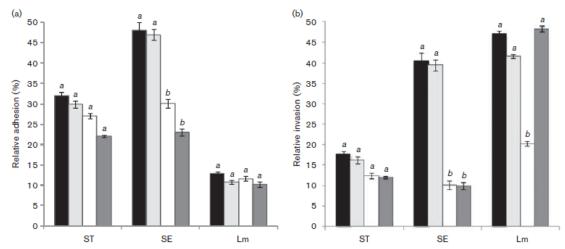

Fig. 2. Competitive exclusion of *Salmonella* strains and *Listeria monocytogenes* after exposure of HT-29 cells to LABs for 3 h: (a) adhesion and (b) invasion. The percentage of relative adhesion (a) and invasion (b) of HT-29 was referred to the corresponding percentage in the absence of pre-exposure to LABs. The data are mean±SD of three independent experiments, each performed in triplicate. For each enteropathogenic strain, different letters above the columns indicate significant differences ($P < 0.05$) between

values. ST: *S.* Typhimurium; SE: *S.* Enteritidis; Lm: *Listeria monocytogenes.* Column shading for LABs used for pre-exposure of HT-29 cell monolayers: black, *Lactobacillus rhamnosus*; light grey, *Lactobacillus paraplantarum*; white, *Lactobacillus plantarum*; dark grey, *Lactobacillus casei*.
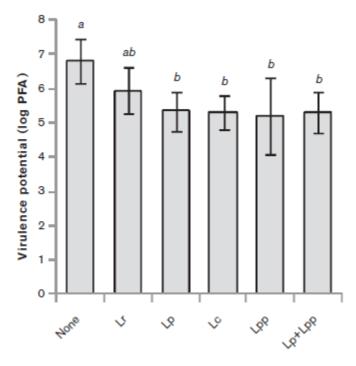

Fig. 3. Virulence potential of *Listeria monocytogenes* strain EGDe without and with previous exposure of HT-29 cells to each LAB. The pathogenic potential of the isolates is expressed as the mean logarithm of the number of plaques formed for 10^7 *Listeria* cells per well (log PFA). The data are mean±SD of three independent experiments, each performed in duplicate. Different letters above the columns indicate significant differences ($P < 0.05$) between values. LABs used for pre-exposure of HT-29 cell monolayers: Lr, *Lactobacillus rhamnosus*; Lp, *Lactobacillus plantarum*; Lc, *Lactobacillus casei*; Lpp, *Lactobacillus paraplantarum*.
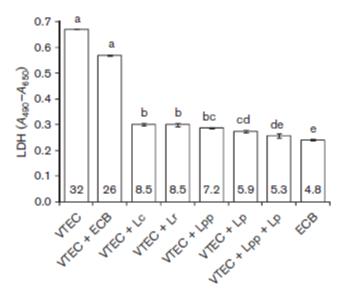
Fig. 4. Cytotoxicity using bacterial CFSC in Vero cells. LDH values are mean±SD of at least three independent experiments, each performed in triplicate. Different letters above the columns indicate significant differences ($P < 0.05$) between values. The percentage of cytotoxicity is indicated within the columns. ECB, *E. coli* B; Lc, *Lactobacillus casei*; Lr, *Lactobacillus rhamnosus*; Lpp, *Lactobacillus paraplantarum*; Lp, *Lactobacillus plantarum*.