# *In vitro* evaluation of the protective role of *Lactobacillus* strains against inorganic arsenic toxicity

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**Abstract**

Inorganic arsenic [iAs, As(III)+As(V)] is considered a human carcinogen. Recent studies show that it has also toxic effects on the intestinal epithelium which might partly explain its systemic toxicity.

The aim of this study is to evaluate the protective role of lactic acid bacteria (LAB) against the toxic effects of iAs on the intestinal epithelium. For this purpose, the human colonic cells Caco-2 were exposed to As(III) in the presence of various LAB strains or their conditioned medium. Results showed that some strains and their conditioned media partially revert the oxidative stress, the production of pro-inflammatory cytokines, the alterations of the distribution of tight junction proteins and the cell permeability increases caused by As(III). These results show that both soluble factors secreted or resulting from LAB metabolism and cell-cell interactions are possibly involved in the beneficial effects. Therefore, some LAB strains have potential as protective agents against iAs intestinal barrier disruption.

**Keywords**: inorganic arsenic; intestinal epithelium; inflammation; oxidative stress; barrier disruption; *Lactobacillus*.

## 1. INTRODUCTION

Drinking water or contaminated foodstuffs are the main sources of dietary arsenic (As) which can be found in different chemical forms with varying degrees of toxicity. Among them, inorganic As [As(III) + As(V)] species are the most toxic. The highest concentrations of inorganic As in foodstuffs are found in seaweeds, some bivalves and rice. For instance, concentrations higher than 100 mg/kg dry weight have been reported in the brown seaweed *Hizikia fusiforme* [1]. Contamination of drinking water by As (up to 1 mg/L) is an endemic problem in some countries [2,3] with levels exceeding the limits recommended by the World Health Organization (0.01 mg/L) [4].

Inorganic As is listed as a human carcinogen [5], and is also associated with other types of pathologies, such as skin lesions, cardiovascular and cerebrovascular diseases, type 2 diabetes and neurobehavioral disorders in children [6]. Gastrointestinal disorders such as dyspepsia, gastroenteritis and chronic diarrhea have been reported in populations chronically exposed to inorganic As [7-9]. Intestinal toxicity associated with As exposure has also been observed in animal models. Rhesus monkeys exposed to As(III) (7.5 mg/kg/day) for 12 months presented acute inflammation of the small intestine and hemorrhage [10]. Animals exposed subchronically (2 months) to As(III) (50 mg/L) through drinking water also present an increase in pro-inflammatory cytokines in the large intestine; this inflammation is accompanied by an increase in intestinal permeability [11].

*In vitro* studies have shown that As(III) induces oxidative stress and a pro-inflammatory response in intestinal epithelial cells, affects the intercellular junctions and increases the permeability through the cell monolayer [12-16]. Disruption of intercellular junctions has a relevant impact on intestinal/systemic toxicity as they maintain the structure of the epithelial layer and the cell polarization and provides an effective barrier against paracellular entry of microorganisms, noxious substances and antigens present in the gastrointestinal lumen into the systemic circulation

[17]. In fact, the pathophysiology of a number of diseases is associated with a dysfunctional intestinal barrier [18]. Considering this, setting up dietary strategies that mitigate damages on the intestinal epithelium associated with As exposure is of great interest, particularly for chronic exposures.

A number of studies have explored different strategies aimed to diminish intestinal mucosa damage. Some of these studies have shown that commensal bacteria and probiotics help to protect the intestinal barrier integrity. For example, *Lactobacillus rhamnosus* GG alleviates the effects of pro-inflammatory cytokines on epithelial barrier integrity, mediated, at least in part, through inhibition of NF-κβ signalling in Caco-2 cells [19]. Probiotics preserve the intestinal barrier in mouse models of colitis and reduce intestinal permeability in human patients with Crohn's disease [20].

On the basis of these previous results, in the present study we have evaluated the possible protective role of various lactic acid bacteria (LAB) strains against the damage induced on Caco-2 cell monolayers exposed to As(III).

## 2. MATERIAL AND METHODS

### 2.1. Chemicals

The As(III) standard (1000 mg/L) was prepared by dissolving 1.320 g of $As_2O_3$ (Riedel de Haën, Seelze, Germany) in 25 mL of KOH 20% m/v (Panreac, Barcelona, Spain), neutralizing with 20% $H_2SO_4$ v/v (Merck, Darmstadt, Germany) and making up to a final volume of 1 L with $H_2SO_4$ 1% v/v.

### 2.2. Lactic acid bacteria

The strains of LAB used were *Lactobacillus acidophilus* BL17 (ATCC4356, American Type Strain Culture Collection, Manassas, VA, USA), *Lactobacillus casei* BL23 [21] and *Lactobacillus brevis* BL36 (ATCC14869). These strains were selected in a preliminary screening of LAB strains

available in our laboratory as they displayed the highest antioxidant and/or anti-inflammatory capacity.

The strains were routinely grown in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) under static conditions at 30 °C (*L. brevis*) or 37 ºC (*L. acidophilus* and *L. casei*) to stationary phase. Cells were harvested by centrifugation (5500×g, 5 min) and washed with phosphate-buffered saline (PBS, Hyclone, Logan, UT, USA). The bacterial pellets were resuspended in PBS and adjusted to different OD (optical density at 595 nm) for subsequent assays.

To obtain the conditioned medium, cells grown in MRS were washed with PBS and suspended at an initial OD of 0.3 in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5 g/L) and L-glutamine (0.6 g/L) (Hyclone) supplemented with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Hyclone) (DMEM-HEPES), and incubated at 30 ºC or 37 ºC for 24 h. Subsequently the cell suspensions were centrifuged (3000×g, 10 min), the supernatants were recovered, sterilized through 0.2 μm filters and stored at -20 °C until use. For the assays of permeability (section 2.6), fetal bovine serum (FBS, Hyclone) was added to the conditioned medium to a final concentration of 7.5% (v/v), to maintain the integrity of the monolayer during the assay.

### 2.3. Cell line culture conditions

The human colon Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, number 86010202, Salisbury, UK). Cells were grown at 37 ºC in an atmosphere with 95% relative humidity and a $CO_2$ flow of 5% in 75 mL flasks with 10 mL of DMEM with high glucose (4.5 g/L) and L-glutamine (0.6 g/L) supplemented with 10% (v/v) of FBS, 1% (v/v) of non-essential amino acids, 1 mM of sodium pyruvate, 10 mM of HEPES, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 0.0025 mg/L of amphotericin B (DMEM-10%FBS). The medium was changed every 2-3 days. When the cell monolayer reached 80% confluence, the cells were detached

with a solution consisting of 0.5 g/L of trypsin and 0.22 g/L of ethylene diamine tetraacetic acid (EDTA), and reseeded at a density of $5 \times 10^4$ cells/cm$^2$. All the reagents used for the cell maintenance were obtained from Hyclone. The assays were performed with cultures between passages 5 to 25.

For As acute exposure (sections 2.4-2.5), Caco-2 cells were seeded in DMEM-10%FBS and 7 days post-seeding, subjected to the following treatments for 24 h:

a. Treatment with DMEM-HEPES (negative control).

b. As treatment: As(III) (1 and 3 mg/L) prepared in DMEM-HEPES.

c. Treatment with conditioned media + As: Conditioned media from LAB strains spiked with As(III) (1 and 3 mg/L).

d. Treatment with strains + As: pre-incubation with LAB (OD 0.3) during 6 h; washing of bacteria with PBS and a subsequent treatment with As(III) (1 and 3 mg/L) prepared in DMEM-HEPES for 24 h.

The selected conditions for the acute exposure were those that had the greatest toxic effect in previous experiments [16].

### 2.4. Determination of the pro-oxidant and pro-inflammatory response

Cells were seeded on 12 well plates in DMEM-10%FBS at a density of $3 \times 10^4$ cells/cm$^2$ and 7 days post-seeding exposed to the treatments described in section 2.3. After exposure, supernatants were recovered and the levels of IL-8 determined using the Human IL-8/CXCL8 ELISA Kit (Sigma, Madrid, Spain) to estimate the pro-inflammatory response. IL-8 levels were normalized per mg of protein, quantified by the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CS, USA).

Cells were used to determine the formation of reactive oxygen (ROS) and/or nitrogen (RNS) species by determining their capacity of oxidizing 2′7′-dichlorofluorescein diacetate (DCFH-DA, Sigma) to dichlorofluorescein (DCF), a fluorescent product. After treatment, cell monolayers were

washed with PBS and incubated (30 min, 37 ºC) with 200 µL 100 µM DCFH-DA dissolved in PBS. Subsequently, the DCFH-DA solution was transferred to a 96-well plate for fluorescence determination. The cells were washed with PBS, and detached with trypsin/EDTA before being lysed with 300 µL of Triton X-100 (Merck, 0.1% w/v in PBS). The samples were sonicated (10 min, 4 ºC) and centrifuged (5700×g, 3 min) and fluorescence was also determined in cell lysate supernatants. DCF concentration was analyzed in a PolarSTAR OPTIMA plate reader (BMG-Labtech, Cary, NC, USA) (λ excitation = 488 nm; λ emission = 530 nm). The fluorescence values obtained were normalized per mg of protein.

### 2.5. Distribution of zonula occludens-1 (ZO-1) in Caco-2 monolayers

Cells were seeded at a density of $4 \times 10^4$ cells/cm$^2$ on round coverslips (10 mm in diameter) placed on 24-well plates and incubated for seven days. Subsequently, cells were subjected to the treatments outlined in section 2.3. After exposure the monolayers were washed with PBS, fixed in 4% (v/v) paraformaldehyde in PBS (Sigma), permeabilized with 0.1% (v/v) Triton X-100 in PBS, and blocked with 10% (v/v) goat serum in PBS (Santa Cruz Biotechnology, Dallas, TX, USA).

For immunolocalization of ZO-1, cells were incubated overnight at 4 °C with rabbit polyclonal anti-ZO1 (ab216880, Abcam, Cambridge, UK) diluted 1:200 in 2% goat serum. For detection, the cells were incubated for one hour at room temperature with goat anti-rabbit Alexa 488 (R37116, Invitrogen, Eugene, OR, USA) diluted 1:200 in 2% goat serum, followed by incubation for 15 min with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) diluted 1:400 in PBS. Finally, the samples were mounted in ProLong® Antifade (Molecular Probes, Eugene, OR, USA) and examined under a Nikon Eclipse 90i epifluorescence microscope.

### 2.6. Determination of cellular permeability

Cells were seeded at a density of $4 \times 10^4$ cells/cm$^2$ on Transwell® inserts (diameter 24 mm, pore

size 0.4 µm, Corning, NY, USA) placed into 6 well plates. The basolateral (lower) compartment was filled with 2 mL of DMEM with high glucose (4.5 g/L) and L-glutamine (0.6 g/L) supplemented with 7.5% (v/v) of FBS, 1% (v/v) of non-essential amino acids, 1 mM of sodium pyruvate, 10 mM of HEPES, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 0.0025 mg/L of amphotericin B (DMEM-7.5%FBS). The apical (upper) compartment was filled with 1.5 mL of DMEM-7.5%FBS with different supplements as outlined below:

a. Treatment with DMEM-7.5%FBS (negative control)

b. As treatment: As(III) (0.1 mg/L) prepared in DMEM-7.5%FBS.

c. Treatment with conditioned media + As: Conditioned media from LAB strains spiked with As(III) (0.1 mg/L).

The plates were incubated for 7 days at 37 °C in an atmosphere with 95% relative humidity and a 5% $CO_2$. The selected conditions for the subchronic exposure (0.1 mg/L As(III), 7 days) was the treatment displaying the highest toxic effect in previous experiments [15]. After incubation, the permeability of the cell monolayers was determined by measuring the transport of Lucifer Yellow (LY) (Sigma). To this end, the solution of LY in DMEM-7.5%FBS was added to the apical compartment at the concentration of 100 µM and incubation was continued for 24 h. Subsequently, aliquots were taken from the basolateral side and the LY content measured by fluorescence in a PolarSTAR OPTIMA plate reader (λ excitation = 488 nm; λ emission = 530 nm).

### 2.7. Statistical analysis

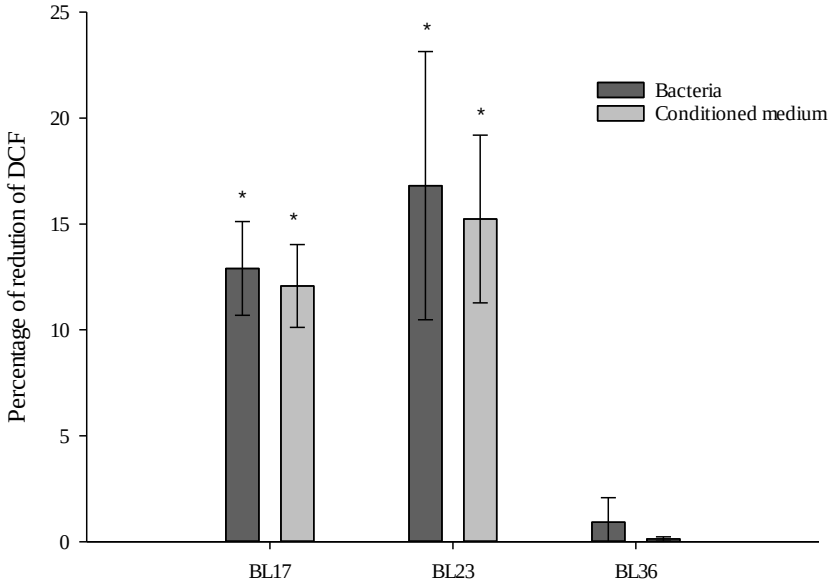
All assays were performed at least in triplicate in independent cultures, and verified in a second experiment. The results were statistically analyzed by one-factor analysis of variance (ANOVA) with Fisher LSD *post hoc* multiple comparison or using the Student t-test (SigmaPlot version 13.0). Differences were considered significant for $p<0.05$.

## 3. RESULTS

### 3.1. Some LAB strains reduce the oxidative stress and the pro-inflammatory response produced by inorganic As

Exposure to 3 mg/L of As(III) resulted in a significant increase of ROS and RNS generation in Caco-2 cultures compared to the untreated cells (up to 29%). The effects of pre-incubation with LAB or co-incubation with their corresponding cell-free conditioned media are shown in Fig. 1. Pre-incubation with cells of strains BL17 and BL23 or co-incubation with their conditioned media resulted in significant decreases in DCF production, indicating a reduced generation of ROS/RNS compared to As(III)-exposed cells. In contrast, pre-incubation with cells of strain BL36 or co-incubation with its conditioned medium had no significant effect.

**Fig. 1. Effect of LAB or their corresponding conditioned media on inorganic As-induced ROS and RNS generation.** Caco-2 cells were exposed to As(III) (3 mg/L) for 24 h after pre-incubation with LAB (0.3 OD) for 6 h, or co-incubated with As(III) (3 mg/L) and conditioned media for 24 h. The bars indicate percentages of decrease in DCF production compared to cells treated with As(III) (3 mg/L) alone (positive control) (mean ± SD, n=4). Asterisks indicate statistically significant differences ($p < 0.05$) with respect to positive controls.



Production of pro-inflammatory interleukin IL-8 by Caco-2 cells significantly increased after As(III) exposure (5-13 fold-changes). Pre-incubation with BL17 or BL23 strains had no significant effects whereas pre-incubation with strain BL36 significantly decreased the production of IL-8 induced by As(III) exposure (Fig. 2). Co-incubation with the conditioned medium from the three strains produced a significant decrease in the production of IL-8 induced by As(III) (BL17: 34-48%; BL23: 29-44%; BL36: 29-44%) (Fig. 3).

**Fig. 2. Effect of pre-incubation with LAB strains on the pro-inflammatory response induced by As(III).** IL-8 levels in the supernatants of Caco-2 cells unexposed (negative control), exposed to As(III) (1 or 3 mg/L) for 24 h (positive control) and pre-incubated with LAB strains BL17, BL23 or BL36 (0.3 OD, 6 h) before As exposure (24 h). Values expressed as pg IL-8/mg protein (mean ± SD, n=4). Asterisks indicate statistically significant differences ($p<0.05$) with respect to their corresponding positive controls.

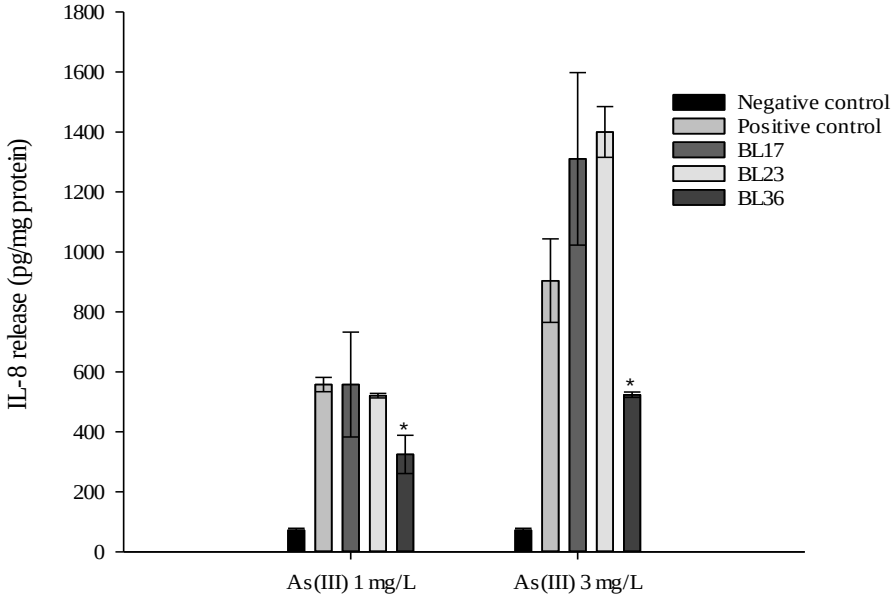
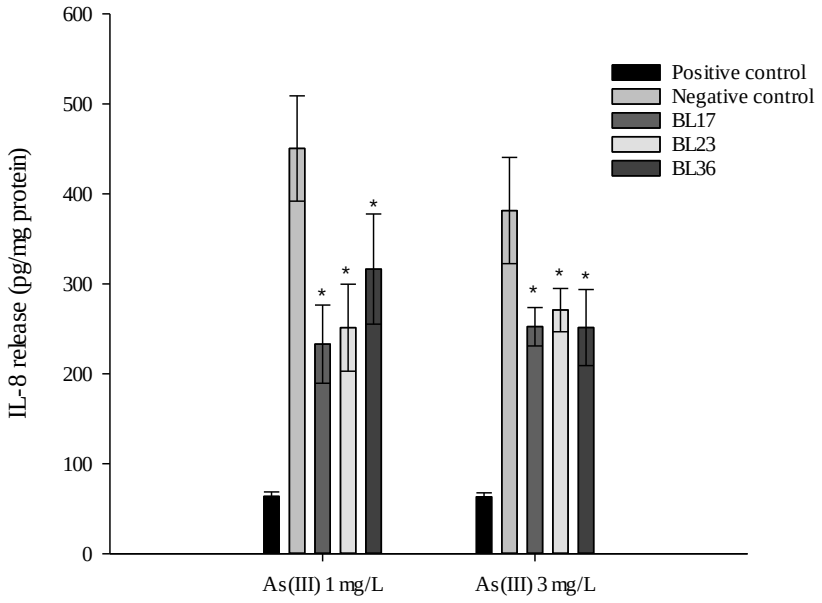


**Fig. 3. Effect of co-incubation with conditioned media from the LAB strains on the pro-inflammatory response induced by As(III).** IL-8 levels in the supernatants of Caco-2 cells unexposed (negative control), exposed to As(III) (1 or 3 mg/L) for 24 h (positive control) and co-incubated with As(III) and LAB conditioned media (from strains BL17, BL23 or BL36) for 24 h. Values expressed as pg IL-8/mg protein (mean ± SD, n=4). Asterisks indicate statistically significant differences ($p < 0.05$) with respect to their corresponding positive controls.



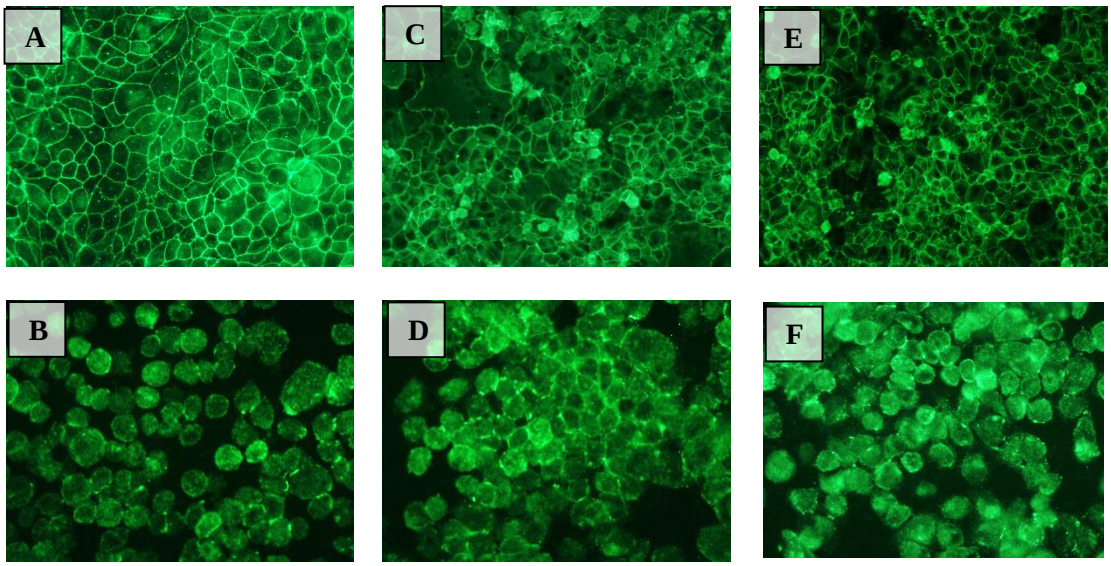

### 3.2. LAB strains alleviate the alterations in ZO-1 protein distribution induced by inorganic As

Exposure to 1 mg/L of As(III) did not result in appreciable changes in the localization of protein ZO-1 (data not shown). However, exposure to 3 mg/L of As(III) resulted in extensive disorganization of the cell monolayer (Fig. 4B) compared to the control (Fig. 4A). Arsenic treated cells presented areas where the monolayer had changes in ZO-1 expression (fluorescence was not observed) or localization (fluorescence appeared in the cytoplasm) (Fig. 4B).

Strain BL17 or its conditioned medium had no appreciable effects on the observed alterations (data not shown). On the contrary, strains BL23 (Fig. 4C) and BL36 (Fig. 4E) and to a lesser extent, their corresponding conditioned media (Fig. 4D and 4F, respectively), partially restored the cobblestone-like structure of the Caco-2 monolayer. Furthermore, ZO-1 protein, especially in

cultures pre-incubated with LAB cells, was mainly located in cell junctions as in the untreated cells (Fig. 4A).

**Fig. 4. Effect of LAB strains and their conditioned medium on the distribution of ZO-1 in Caco-2 cells exposed to As(III).** A, untreated cells; B, cells exposed to 3 mg/L As(III) for 24 h; C, cells pre-incubated 6 h with BL23 and exposed to As(III) for 24 h; D, cells co-incubated with BL23 conditioned medium and As(III) for 24 h; E, cells pre-incubated 6 h with BL36 and exposed to As(III) for 24 h; F, cells co-incubated with BL36 conditioned medium and As(III) for 24 h. Magnification 20×.



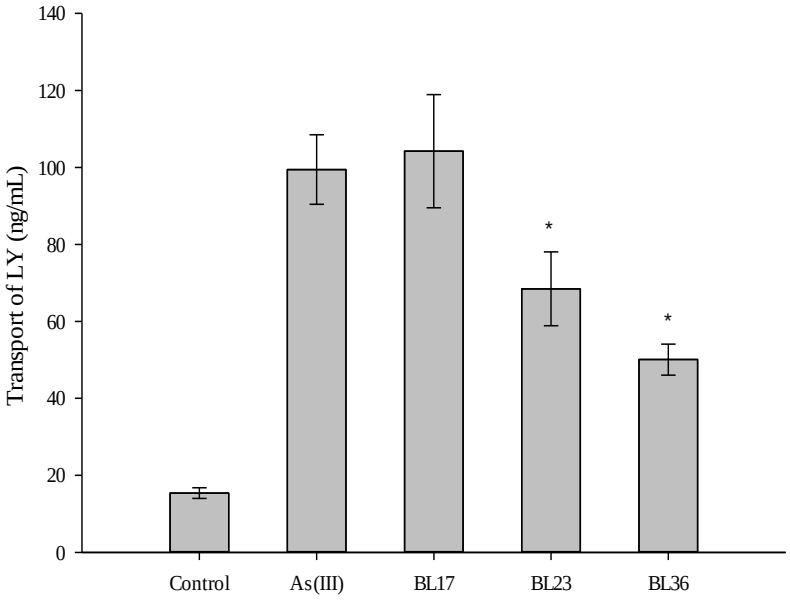

### 3.3. LAB strains diminish the increase of the monolayer permeability produced by inorganic As

The effect of As(III) (0.1 mg/L) on the permeability across Caco-2 cell monolayers was determined using a subchronic exposure (7 days). Extended incubation with LAB strains resulted in acidification of the medium that led to disorganization of the cell monolayers. For this reason, only conditioned media were used for these assays.

After seven days of exposure to As(III) 0.1 mg/L, the transport of LY to the basolateral compartment increased considerably compared to that found in untreated cells (Fig. 5), thus

indicating an increased paracellular permeability of the cell monolayers. Co-incubation with conditioned media from strains BL23 and BL36 resulted in significant reductions in LY permeability compared to cells exposed to As(III), whereas conditioned medium from strain BL17 had no effect (Fig. 5).

**Fig. 5. Effect of LAB conditioned medium on the alterations of Caco-2 monolayers permeability caused by inorganic As.** Transport of Lucifer yellow (LY, 24 h) in control cells, cells exposed to 0.1 mg/L As(III) for 7 days and cells co-incubated with the conditioned media (from BL17, BL23 and BL36) and 0.1 mg/L As(III) for 7 days. Values expressed as ng LY/mL (mean ± SD, n=3). Asterisks indicate statistically significant differences ($p < 0.05$) with respect to the cells exposed to As(III).




## 4. DISCUSSION

Effectiveness of numerous compounds, some of them of food origin, in reducing the toxicity of inorganic As has been evaluated previously [22]. Most of the studies show that food components can counteract the oxidative stress generated by inorganic As [23-25]. The effectiveness of some compounds has also been proved in humans. Biswas et al. [26] showed that consumption of a turmeric supplement for 3 months reduced DNA damage, retarded ROS generation, and reduced

lipid peroxidation in chronically exposed populations. The use of LAB to alleviate the effects of other toxic trace elements and chemical contaminants has been previously explored in a number of studies [27], as they have the advantage of being easily administered via food preparations and to possess high consumer acceptance. However, no previous studies have dealt with the use of LAB to protect against inorganic As toxicity. LAB can effectively remove trace elements such as lead (Pb) and cadmium (Cd) from the surrounding medium [28,29], but their ability to eliminate inorganic As is negligible [30]. Indeed, we have assayed the uptake of As(III) (1 and 0.1 mg/L, 4 h) by the three strains used in the present study, finding a low binding capacity (<7%) (unpublished data). This limited interaction may be related to the low affinity for the negatively charged forms of inorganic As due to the net negative charge of LAB cell envelopes [30]. Therefore, the alleviation of inorganic As toxicity reported here is unlikely related to sequestration of As from the medium. Furthermore, the fact that the bacteria-free conditioned medium has similar protective effects points to bacteria-derived products/metabolites as one of the responsible factors. Soluble compounds present in supernatants from *Lactobacillus* cultures have been shown to mediate probiotic properties in other situations [31-33].

Interestingly, the effect of LAB strains varied depending on the toxicity end-point evaluated. Cells and conditioned media of strains BL17 and BL23 decreased the production of ROS/RNS induced by As(III), whereas strain BL36 had no significant effect. On the other hand, only BL36 cells significantly decreased IL-8 production in response to As(III); however the conditioned medium from the three strains reduced this pro-inflammatory response. The use of conditioned medium was generally more effective than live bacteria. This was probably due to acidification of the medium by bacteria during the treatment time (up to 24 h).

The ability of some *Lactobacillus* strains to decrease oxidative stress or pro-inflammatory response in animals treated with toxic metals (e.g. Pb and Cd) has been reported [28,34,35], but the mechanisms of action are not clearly determined. A low Mw peptide present in *L. rhamnosus* GG

conditioned supernatants induced the expression of Hsp25 and Hsp72 in epithelial cells, thus mediating its protective effect against oxidative damage [33]. The ability of some *Lactobacillus* strains and their derived molecules to act as immunomodulators has been widely studied [36,37]. This effect has been related to inhibition of the activation of the nuclear factor NF-κβ [38,39] or activation of the peroxisome proliferator-activated receptor gamma (PPARγ) [40,41].

Reduced oxidative stress and pro-inflammatory cytokines expression may be at the basis of the observed maintenance of the monolayer structure induced by lactobacilli after As(III) exposure, although the above discussed discrepancies among strains probably indicate different modes of action. As the permeability of intestinal epithelial cell monolayers mostly depends on tight junctions, the effect of LAB strains on ZO-1 distribution may account for the observed decrease in LY transport across the monolayers in cells injured by As(III). A protective effect of some LAB strains on the epithelial barrier has also been previously observed in cellular models exposed to contaminants. Karczewski et al. [42] showed that pretreatment of Caco-2 monolayers with *L. plantarum* significantly attenuated the effects of phorbol ester-induced dislocation of ZO-1. Similarly, *Lactobacillus plantarum* CCFM8610 protected against changes in the distribution of ZO-1 and Claudin-1 tight junction proteins in HT-29 cells exposed to Cd, enhancing the intestinal barrier [35].

In summary, our results show that LAB strains modulate a number of protective and structure-maintenance mechanisms in Caco-2 cells exposed to As(III). These effects are possibly mediated by soluble factors; as conditioned media were sufficient to induce protection. This is the first study that demonstrate a protective effect of LAB strains against toxic effects induced by As(III). The fact that soluble factors induce these protective effects indicates that they have potential for application to affected populations as the use of live bacteria would not be required. Additional studies are under way to confirm these results *in vivo*.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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