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## ***Lactobacillus delbrueckii* subsp *lactis* CIDCA 133 modulates response of human epithelial and dendritic cells infected with *Bacillus cereus***

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### RESEARCH ARTICLE

#### Abstract

It is known that probiotic microorganisms are able to modulate pathogen virulence. This ability is strain dependent and involves multiple interactions between microorganisms and relevant host's cell populations. In the present work we focus on the effect of a potentially probiotic lactobacillus strain (*Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133) in an *in vitro* model of *Bacillus cereus* infection. Our results showed that infection of intestinal epithelial HT-29 cells by *B. cereus* induces nuclear factor kappa B (NF- $\kappa$ B) pathway. Noteworthy, the presence of strain *L. delbrueckii* subsp. *lactis* CIDCA 133 increases stimulation. However, *B. cereus*-induced interleukin (IL)-8 production by epithelial cells is partially abrogated by *L. delbrueckii* subsp. *lactis* CIDCA 133. These findings suggest that signalling pathways other than that of NF- $\kappa$ B are involved. In a co-culture system (HT-29 and monocyte-derived dendritic cells), *B. cereus* was able to translocate from the epithelial (upper) to the dendritic cell compartment (lower). This translocation was partially abrogated by the presence of lactobacilli in the upper compartment. In addition, infection of epithelial cells in the co-culture model, led to an increase in the expression of CD86 by dendritic cells. This effect could not be modified in the presence of lactobacilli. Interestingly, infection of enterocytes with *B. cereus* triggers production of proinflammatory cytokines by dendritic cells (IL-8, IL-6 and tumour necrosis factor alpha (TNF- $\alpha$ )). The production of TNF- $\alpha$  (a protective cytokine in *B. cereus* infections) by dendritic cells was increased in the presence of lactobacilli. The present work demonstrates for the first time the effect of *L. delbrueckii* subsp. *lactis* CIDCA 133, a potentially probiotic strain, in an *in vitro* model of *B. cereus* infection. The presence of the probiotic strain modulates cell response both in infected epithelial and dendritic cells thus suggesting a possible beneficial effect of selected lactobacilli strains on the course of *B. cereus* infection.

**Keywords:** probiotics, epithelial cells, immunomodulation, virulence, dendritic cells

#### 1. Introduction

Probiotic microorganisms have long been proposed to promote human health (Ashraf and Shah, 2014; Marchesi *et al.*, 2015; Martin *et al.*, 2014). Lactobacilli and bifidobacteria species are widely used to prevent and treat allergy and intestinal disorders (Tojo *et al.*, 2014). Although several authors have shown favourable effects of probiotics in human health, their mechanism of action is not completely

understood. Some proposed mechanisms are modulation of enzymatic activities (Hugo *et al.*, 2006; Parvez *et al.*, 2006), inhibition of intestinal pathogens (Franco *et al.*, 2013), modulation of host defence mechanisms (Dogi *et al.*, 2010; Galdeano and Perdigón, 2006; Kang and Im, 2015; Linares *et al.*, 2015; Riedel *et al.*, 2006; Zeuthen *et al.*, 2006), immune regulation through balance of pro-inflammatory and anti-inflammatory cytokines (Hua *et al.*, 2010), and competition

for adhesion sites at the intestinal epithelium (Jung *et al.*, 2015; Scaldaferrri *et al.*, 2012; Viggiano *et al.*, 2015).

Intestinal epithelial cells (IECs) represent an important barrier between lamina propria cells and the potentially harmful luminal contents. IECs are important immunoeffector cells with the capacity to release cytokines, chemokines, and other molecules involved in antigen presentation and immune defence (Canny *et al.*, 2006). IECs and antigen presenting cells such as dendritic cells (DC) and macrophages play a key role in the orchestration of immune responses. Mucosal DCs are highly specialised in function and display a unique response to toll-like receptor ligands, are capable of driving immunoglobulin isotype switching to IgA, can imprint gut-homing receptors on T and B cells, and drive either T regulatory or Th17 cells depending on the analysed subtype. In the last years, there are several reports describing how the local microenvironment can shape DC function (Rescigno, 2010). Immunomodulation by probiotics can be achieved by modification of the Th1/Th2 balance (Hua *et al.*, 2010). Furthermore, they can induce B cells migration and increase specific immunoglobulin A (IgA) secretion by modulation of intestinal DC (Macpherson and Uhr, 2004), thus facilitating antigen uptake and presentation in Peyer's patches.

The nuclear factor kappa B (NF- $\kappa$ B) is a transcription factor which plays a key role in regulating biological processes. NF- $\kappa$ B is typically present and resides in the cytoplasm of most cells as a complex with members of the I $\kappa$ B inhibitor protein family. In response to signals, NF- $\kappa$ B activation occurs via phosphorylation of its inhibitor, which dissociates from the NF- $\kappa$ B dimer allowing the translocation to the nucleus, inducing gene expression. Over 200 physiological stimuli are known to activate NF- $\kappa$ B, for instance, proinflammatory cytokines; bacterial toxins; viral products; and cell death stimuli (Baldwin, 1996; Tergaonkar, 2006). In this context, commensal bacteria or probiotic microorganisms, mostly through Toll like receptor-mediated signalling, could modify activation of NF- $\kappa$ B of intestinal epithelial cells (Cerf-Bensussan and Gaboriau-Routhiau, 2010). On the other hand, intestinal infections caused by enterobacteria, induce activation of an inflammatory cascade that lead to an increase of proinflammatory mediators (e.g. interleukin (IL)-8) that in turn enhances cell recruitment to the infection zone. Interestingly, some probiotic microorganisms are capable to inhibit IL-8 production from intestinal epithelial cells through modulation of NF- $\kappa$ B (Sokol *et al.*, 2008). IL-8 is a pro-inflammatory interleukin that plays a pivotal role in cell recruitment to the site of infection. It is a relevant marker of intestinal inflammation (Roberts-Thomson *et al.*, 2011) and IL-8 levels has been assessed in studies of the immunomodulatory effect of potentially probiotic strains (Kechaou *et al.*, 2013; Torres-Maravilla *et al.*, 2015). Interestingly, even proinflammatory strains lead

to protective effects (Kechaou *et al.*, 2013). Other authors propose that probiotics contribute to intestinal homeostasis through stimulation of tumour necrosis factor alpha (TNF- $\alpha$ ) production, whereby beneficial effects would be associated with immunological stimulation process (Mizoguchi *et al.*, 2008; Pagnini *et al.*, 2010).

*Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 is a potentially probiotic strain. Indeed, this strain has the ability to inhibit microorganisms involved in food-spoilage (Kociubinski *et al.*, 1996), to resist bile (Kociubinski *et al.*, 1999), to antagonise biological effects of enterohaemorrhagic *Escherichia coli* (EHEC) on cultured eukaryotic cells (Hugo *et al.*, 2008) and to inhibit harmful enzymatic activities (Hugo *et al.*, 2006). Furthermore, this strain has differential susceptibility to enterocyte-derived antimicrobial peptides (Hugo *et al.*, 2010).

*Bacillus cereus* is a spore-forming microorganism responsible for foodborne illness, i.e. emetic and diarrhetic syndromes (Bottone, 2010). Virulence of *B. cereus* is a multifactorial process that involves regulation of the expression of relevant virulence coding genes for extracellular factors (Gohar *et al.*, 2002) as well as the ability of adhesion/invasion events (Minnaard *et al.*, 2001, 2004, 2007; Rowan *et al.*, 2001). We have previously isolated four *B. cereus* strains that show differences in the presence of sequence of virulence genes as well as different biological effects on eukaryotic cells (Minnaard *et al.*, 2004, 2007, 2013).

In this work, we studied the effect of *L. delbrueckii* subsp. *lactis* CIDCA 133 on *B. cereus* infection of human epithelial and DC. In this context, HLA and CD86 markers from DC, cytokine production and NF- $\kappa$ B activation on epithelial cells were evaluated.

## 2. Materials and methods

### Bacterial strains and culture conditions

*L. delbrueckii* subsp. *lactis* strain CIDCA 133 belongs to the CIDCA culture collection. Lactobacilli were grown in De Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) at 37 °C for 16 h in anaerobic conditions. Microorganisms, stored frozen at -80 °C, were reactivated twice in liquid medium before the assays. *B. cereus* strains were cultured in brain heart infusion (BHI) broth (Biokar Diagnostics) supplemented with glucose 0.1% (w/v) (BHIG). Bacteria were cultured for 16 h at 32 °C under orbital agitation and afterwards, they were inoculated (4% v/v) in 5 ml of BHIG and incubated with agitation at 32 °C for 3 h. Microorganisms were harvested by centrifugation (900×g for 10 min). *B. cereus* strains 2 and M2, were isolated from infant formula (Minnaard *et al.*, 2001), T1 (Buchanan and



Schultz, 1992) and B10502 (Minnaard *et al.*, 2004) were involved in different foodborne outbreaks.

A correlation curve between absorbance measured at 600 nm ( $A_{600\text{nm}}$ ) versus cfu was plotted for each strain. The  $A_{600\text{nm}}$  values were employed to calculate the bacterial number used in each experiment.

### Labelling of bacteria

Bacteria labelling was carried out with carboxyfluorescein diacetatesuccinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) at a final concentration of 5  $\mu\text{mol/l}$  (Tuominen-Gustafsson *et al.*, 2006). Microorganisms were incubated for 30 min at 37 °C in the dark, and washed twice with phosphate-buffered saline (PBS).

### Cell lines and culture medium

HT-29 (ATCC<sup>®</sup> HTB-38<sup>™</sup>) and HT-29-NF- $\kappa$ B-hrGFP reporter cells (Guimarães *et al.*, 2010) were cultured in DMEM or RPMI 1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) foetal bovine serum (FBS) (Life Technologies). HT-29-NF- $\kappa$ B-hrGFP cells were obtained after stable transfection of HT-29 cells with the pNF- $\kappa$ B-hrGFP plasmid from the PathDetect Signal Transduction Pathway cis-Reporting Systems Kit (Stratagene, Santa Clara, CA, USA). Briefly, subconfluent HT29 cells were transfected with pNF- $\kappa$ B-hrGFP plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and selected with hygromycin. After two weeks of selection, cells were stimulated for 24 h with a pro-inflammatory cocktail (25 ng/ml TNF- $\alpha$ , 1.25 ng/ml IL-1b and 3.75 ng/ml interferon gamma (IFN- $\gamma$ )) and GFP positive cells were sorted with a MoFlo cell sorter (Dako, Carpinteria, CA, USA).

Cells were routinely propagated in 25 or 75  $\text{cm}^2$  tissue culture flasks at 37 °C, 5%  $\text{CO}_2$  in a humidified incubator. Subsequently, cells were trypsinised and concentration was adjusted in order to obtain the required cell concentrations (see below). In all the assays cells were cultured for less than twenty passages.

### Generation of human monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PBMC) were obtained as described by Tiscornia *et al.*, (Tiscornia *et al.*, 2012) by Ficoll-Hypaque density-gradient centrifugation and stored in liquid nitrogen before use. For DCs differentiation, monocytes were incubated during 48 h in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS, 1000 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ, USA) and IL-4 (1% of a conditioned supernatant from the IL-4 transfected J588L cell line). Monocyte-derived DCs were harvested and cultured in 24-well tissue culture plates for experiments.

Cell purity was assessed by cell surface staining and flow cytometry using antibodies (described below in flow cytometry and antibodies). Viability was determined by trypan blue exclusion. The selection criterion for sample acceptance was established in order to choose donors presenting more than 85% of CD11c<sup>+</sup>/CD14<sup>-</sup> cells and less than 5% of lymphocyte contamination (CD3<sup>+</sup> and/or CD19<sup>+</sup> cells) after the DC-differentiation process. Cells with the following phenotype (evaluated by flow cytometry): CD11c<sup>+</sup>CD14<sup>-</sup>MHC-II<sup>low</sup>CD86<sup>low</sup> were considered immature DCs, and cells expressing CD11c<sup>+</sup>CD14<sup>-</sup>MHC-II<sup>high</sup>CD86<sup>high</sup> were considered mature DCs.

### NF- $\kappa$ B activation assay using HT-29-NF- $\kappa$ B-hrGFP reporter cells

250  $\mu\text{l}$  of cell suspension containing  $2.5 \times 10^5$  cells/ml were added per well in 24-well tissue culture plates. Stimulation with *B. cereus* and *L. delbrueckii* subsp. *lactis* CIDCA 133 was performed using a multiplicity of infection of 20 for 18 h. Culture medium (DMEM) was supplemented with the bacteriostatic antibiotic chloramphenicol (100  $\mu\text{g/ml}$ ) to prevent bacterial growth during the experiment (Minnaard *et al.*, 2004). TNF- $\alpha$  (3 ng/ml) stimulated cells were used as a positive control. Following infection, supernatant was obtained, clarified by centrifugation and stored at -80 °C for IL-8 determination. Cells were washed twice with PBS and detached with trypsin. Green fluorescent protein (GFP) expression was analysed by flow cytometry (Cyan<sup>™</sup> ADP Analyzer; Beckman Coulter, Brea, CA, USA). For each sample, 10,000 counts were recorded, which were gated on a forward scatter (FSC) versus side scatter (SSC) dot plot. Doublets were excluded from analysis.

### Co-culture assays

In the present study we have used an experimental setting previously optimised in our lab (Grompone *et al.*, 2012; Tiscornia *et al.*, 2012) and by other groups (Mileti *et al.*, 2009; O'Hara *et al.*, 2006). Briefly, human monocyte-derived DCs were seeded ( $1.25 \times 10^5$  cells/well) in 12-well tissue culture plates (Corning Inc., Corning, NY, USA) using 500  $\mu\text{l}$  of culture medium (RPMI 1640). Non-polarised (HT-29 or HT-29-NF- $\kappa$ B-hrGFP according to the experiment) cells were grown in the upper chamber of a transwell filter (3  $\mu\text{m}$  diameter of pores; Corning Costar, Cambridge, MA, USA) and incubated for 2 days in RPMI 1640 supplemented with 10% heat-inactivated FCS. Inserts containing epithelial cells (HT-29 or HT-29-NF- $\kappa$ B-hrGFP according to the experiment, see below) monolayer were transferred to the 12-well plates containing DCs. Fresh complete culture medium was added (500  $\mu\text{l}$ ) to the upper compartment and the cells were incubated for further 6 h.

The apical surface of the monolayers was stimulated with a bacteria-cell ratio of 20:1. Non-stimulated cells and cells

treated with 0.5 µg/ml of lipopolysaccharide (LPS) from *E. coli* serotype O26:B6 (Sigma-Aldrich, St. Louis, MO, USA) were used as controls. Plates were incubated for 18 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. Culture supernatants from the basal compartment were clarified by centrifugation and stored at -80 °C for cytokines determination. Monocyte-derived DCs were harvested and stained for flow cytometry analyses.

In order to evaluate the effect of *B. cereus* in a co-culture system experiments with DCs and HT-29 cells were performed. HT-29 cells cultured in the upper compartment were apically infected with CFSE-labelled *B. cereus* strain B10502 and incubated for 18 h in RPMI 1640 with 100 µg/ml chloramphenicol. After washing with PBS, cells were detached with trypsin and analysed by flow cytometry to assess association. Similarly, association with DC in the lower compartment was determined.

All the experiments were performed in the presence or absence of non-labelled strain CIDCA 133 in the upper compartment.

### Flow cytometry and antibodies

The following antibodies were used for flow cytometry: B-ly6 (anti-human CD11c, allophycocyanin (APC)-conjugated), 2331 (anti-human CD86, phycoerythrin (PE)-conjugated), TU36 (anti-human HLA-DR fluorescein isothiocyanate (FITC)-conjugated), M5E2 (anti-human CD14 FITC-conjugated), HIB19 (anti-human CD19 PE-conjugated), HIT3a (anti-human CD3 FITC-conjugated). The corresponding isotype controls were used. All antibodies were used according to manufacturer's instructions (BD Pharmingen, San Jose, CA, USA). Surface markers (HLA-DR and CD86) were expressed as the median of the fluorescence intensity (MFI) according to Tiscornia *et al.* (2012). For sample analysis, 10,000 counts, gated on FSC vs SSC dot plot, were recorded. Acquisitions were performed using a Cyan™ ADP (Beckman Coulter) flow cytometer and Summit 4.3 software.

### Cytokine quantification

IL-6, IL-8, IL-10, IL-12 p70 and TNF-α levels were determined by FlowCytomix™ technology (Bender MedSystems, Vienna, Austria) and analysed by flow cytometry (BMS FlowCytomix Software version 2.2.1).

### Statistical analysis

Results were obtained from 2 or 3 independent experiments. Student t-test and median analysis were performed with InfoStat (InfoStat, Version 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Córdoba, Argentina).

Differences were considered statistically significant when  $P < 0.05$ .

## 3. Results

### *Bacillus cereus* induced NF-κB activation in HT-29-NF-κB-hrGFP reporter cells

In order to study the effects of *B. cereus* strains on NF-κB activation, HT-29-NF-κB-hrGFP reporter cells were incubated with microorganisms for 18 h and the activation of NF-κB was determined by flow cytometry (% of GFP<sup>+</sup> cells). A significant increase ( $P < 0.05$ ) of GFP<sup>+</sup> cells was found in cells infected with *B. cereus* (Figure 1);  $4.1 \pm 0.1\%$ ;  $3.9 \pm 0.5\%$ ;  $3.6 \pm 0.3\%$  and  $4.0 \pm 0.0\%$  for strain B10502, T1; M2 and 2, respectively. Interestingly, the co-incubation of *L. delbrueckii* subsp. *lactis* strain CIDCA 133 with *B. cereus* (strains B10502, T1 and M2) led to a further increase of GFP<sup>+</sup> cells ( $P < 0.005$ ) compared with cells infected only with *B. cereus* strains ( $6.9 \pm 0.3\%$ ;  $9.8 \pm 0.1\%$  and  $8.3 \pm 0.2\%$  for strains B10502, T1 and M2, respectively). On the other hand, the *Lactobacillus* had no effect on cells infected with *B. cereus* strain 2 ( $3.8 \pm 0.7\%$ ). The positive control (TNF-α-stimulated cells) led to  $33.4 \pm 1.5\%$  of GFP<sup>+</sup> cells (Figure 1).

### *Bacillus cereus* induced IL-8 production by HT-29-NF-κB-hrGFP reporter cells

Since several cis elements, including a binding site for the inducible NF-κB, have been identified in the regulatory region of the IL-8 gene, we have examined the ability of different *B. cereus* strains to stimulate IL-8 secretion. This cytokine production was significantly increased when HT-29-NF-κB-hrGFP reporter cells were infected with B10502, T1 and 2 strains (Figure 2). IL-8 levels after stimulation with B10502 strain ( $2,009.7 \pm 131.8$  pg/ml) were comparable to those obtained in TNF-α-stimulated cells ( $2,063.8 \pm 141.6$  pg/ml), while after T1 and 2 strains stimulation were  $796.8 \pm 64.0$  pg/ml and  $782.7 \pm 44.4$  pg/ml, respectively. In contrast, IL-8 levels produced by cells incubated with *B. cereus* M2 strain ( $197.5 \pm 29.6$  pg/ml) or *L. delbrueckii* subsp. *lactis* strain CIDCA 133 ( $143.7 \pm 18.9$  pg/ml) were slightly higher than levels produced by control unstimulated cells ( $52.7 \pm 11.0$  pg/ml) (Figure 2).

Interestingly, the presence of the *Lactobacillus* strain significantly ( $P < 0.05$ ) reduced the IL-8 production of *B. cereus* infected cells. Indeed,  $562.2 \pm 27.7$ ,  $193.6 \pm 2.3$  and  $232.8 \pm 8.6$  pg/ml were found for strains B10502, T1 and 2, respectively (Figure 2). On the other hand, *Lactobacillus* did not change the IL-8 production of M2-stimulated cells ( $166.6 \pm 72.6$  pg/ml).

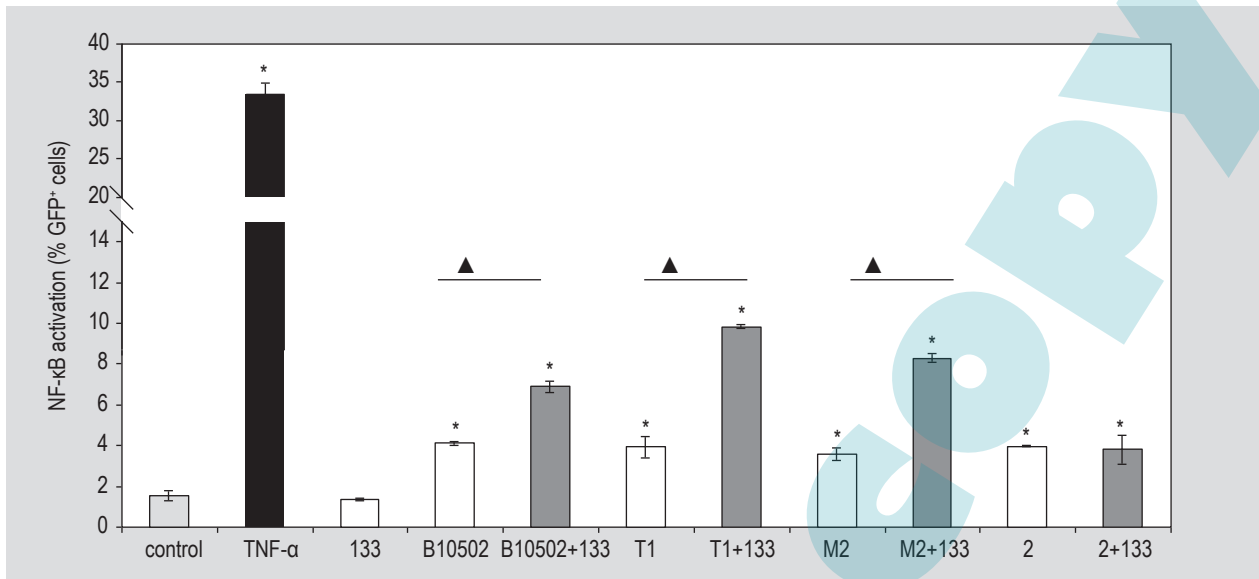


Figure 1. NF- $\kappa$ B activation in HT-29-NF- $\kappa$ B-hrGFP cells infected with different strains of *Bacillus cereus* in the presence or not of *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133. Different microorganisms (*L. delbrueckii* subsp. *lactis* CIDCA 133 or *B. cereus* strains: B10502, T1, M2 and 2) were added to the cells and incubated for 18 h. NF- $\kappa$ B activation (measured by the percentage of GFP+ cells) was analysed by flow cytometry. Stimulation with TNF- $\alpha$  was used as a positive control. Bars represent means  $\pm$  standard deviation from three independent experiments.  $\blacktriangle$  indicate significant differences ( $P < 0.005$ ) between the bars; \* indicate significant differences with control.

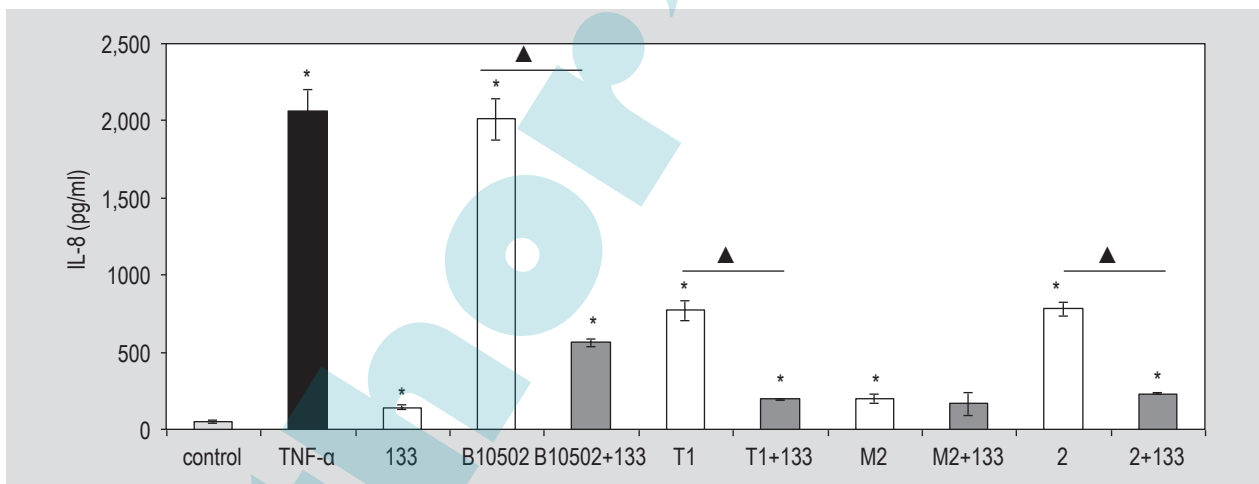


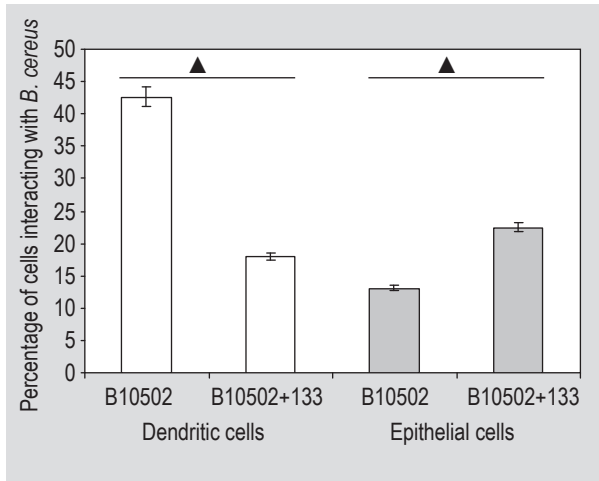
Figure 2. Effect of *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 on IL-8 production induced by *Bacillus cereus* strains in HT-29-NF- $\kappa$ B-hrGFP cells. Different microorganisms (*L. delbrueckii* subsp. *lactis* CIDCA 133 or *B. cereus* strains: B10502, T1, M2 and 2) were added to HT-29-NF- $\kappa$ B-hrGFP cells and incubated for 18 h. IL-8 production was measured on supernatants by flow cytometry. Stimulation with TNF- $\alpha$  was used as a positive control. Bars represent means  $\pm$  standard deviation from three independent experiments.  $\blacktriangle$  indicate significant differences ( $P < 0.005$ ); \* indicate significant differences with control.

### *Lactobacillus delbrueckii* modifies *Bacillus cereus* interaction with dendritic and HT-29 cells

Infection of HT-29 cells (upper compartment) in co-culture with monocyte-derived DC (lower compartment) was performed in order to assess association of bacteria to eukaryotic cells. These studies were performed with strain B10502 since this strain was able to induce strong

inflammatory response in HT-29 cells (IL-8 production) and lactobacilli were capable to significantly decrease this effect.

As depicted in Figure 3, *B. cereus* strain B10502 was able to migrate from the upper compartment to the lower compartment. In addition, the presence of lactobacilli modified the percentage of *B. cereus* associated to DC. Indeed,  $42.7 \pm 2.0\%$  of the DC were associated to *B. cereus*



**Figure 3.** Association of CFSE-labelled *Bacillus cereus* B10502 to monocyte-derived dendritic cells and epithelial cells (HT-29). Association of *B. cereus* B10502 to monocyte-derived dendritic cells (white bars) and epithelial cells (grey bars) after 18 h of incubation in the presence or not of *Lactobacillus delbrueckii* subsp *lactis* CIDCA 133. Microorganisms were added to the upper compartment (HT-29 cells) in a co-culture system with dendritic cells (DC) in the lower compartment. Bars represent means  $\pm$  standard deviation from two independent experiments.  $\blacktriangle$  indicate significant differences between compared treatments ( $P<0.05$ ).

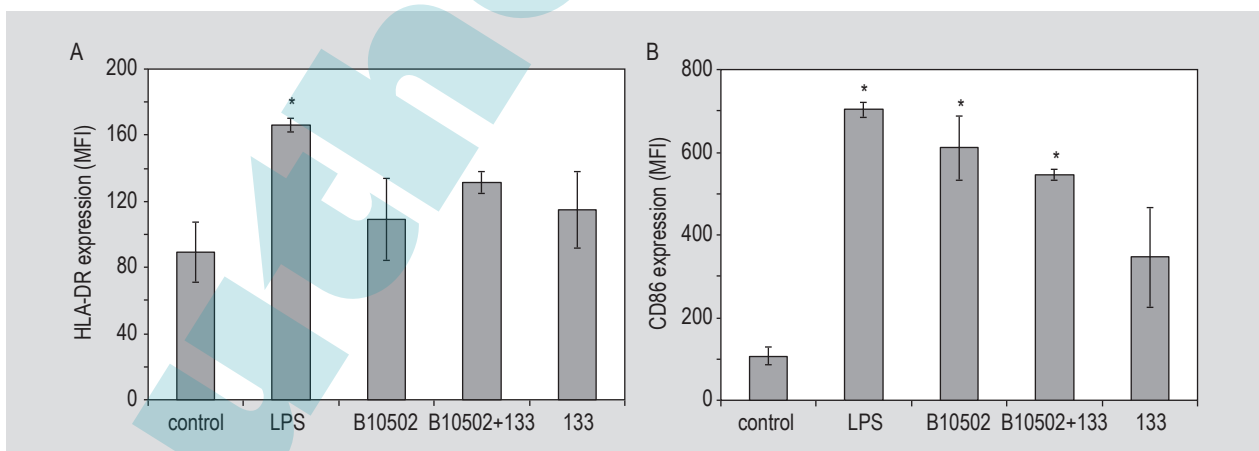
whereas these values decreased to  $18.0\pm 0.6\%$  in the presence of strain 133. In contrast, HT-29 cells (upper compartment) revealed different association pattern:  $13.1\pm 0.4\%$  of cells interacting with *B. cereus* while  $22.4\pm 0.7\%$  of cells were found when the lactobacilli was present during infection.

### CD86 and HLA-DR expression markers on dendritic cells

In order to determine the DC response in co-culture with HT-29 cells to the bacteria, CD86 and HLA-DR expression markers were assessed after 18 h of incubation with *B. cereus* strain B10502 alone or in combination with *L. delbrueckii* subsp. *lactis* strain CIDCA 133. As shown in Figure 4, even though the system was responsive to LPS stimulation, no differences in HLA-DR expression levels (control MFI:  $89.1\pm 18.1$ ) were observed in cells stimulated with *B. cereus* B10502 (MFI:  $108.9\pm 24.8$ ), lactobacilli (MFI:  $114.6\pm 23.3$ ), or both microorganisms (MFI:  $131.2\pm 6.7$ ) when compared to the control (cells without stimulation). In contrast, expression of the co-stimulatory marker CD86 induced by *B. cereus* B10502 was similar to the positive control stimulated with LPS (MFI  $611.1\pm 77.8$  and  $703.3\pm 18$ , respectively). Incubation of cells with *L. delbrueckii* subsp. *lactis* strain CIDCA 133 alone lead to a trend of higher expression of CD86 ( $P=0.11$ ) as compared with control unstimulated cells.

### Cytokine production by dendritic and HT-29 cells in co-culture with *Bacillus cereus*

To determine secretion of cytokines, supernatants of co-cultures were analysed after 18 h of incubation with *B. cereus* strain B10502 alone or in combination with *L. delbrueckii* subsp. *lactis* strain CIDCA 133. Incubation with single strains as well as with combined strains lead to a significant stimulation of IL-8 production in co-cultures as compared with unstimulated control ( $885.66\pm 19.28$  pg/ml). Although these values were lower than those obtained for the LPS control ( $6,181.4\pm 65.6$  pg/ml) there were no



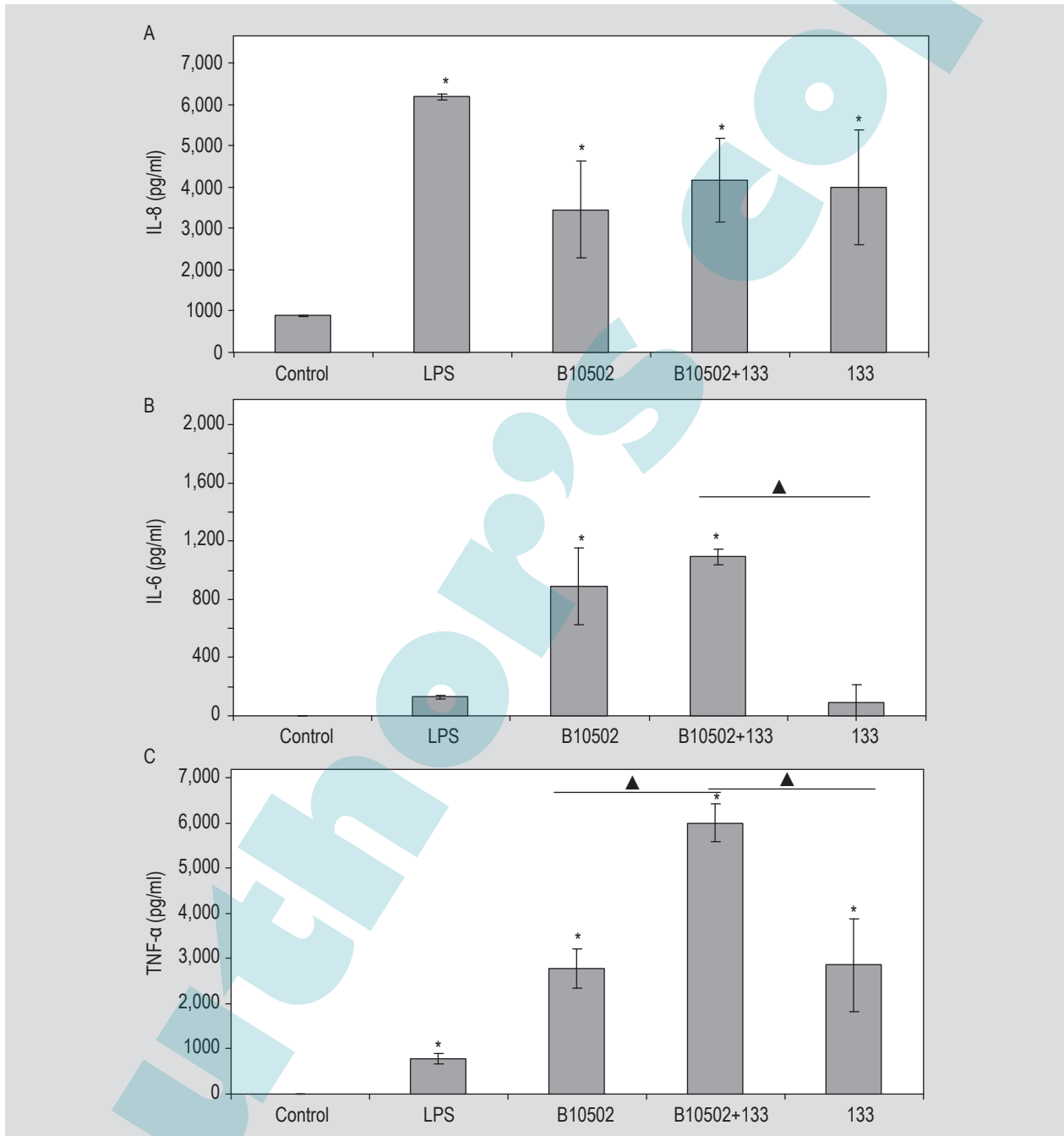
**Figure 4.** Expression of (A) HLA-DR and (B) CD86 in monocyte-derived dendritic cell (DC) co-cultured with HT-29-NF- $\kappa$ B-hrGFP cells, *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 and *Bacillus cereus* B10502. Epithelial cells (upper compartment) were infected with *B. cereus* B10502 in the presence or absence of *L. delbrueckii* subsp *lactis* CIDCA 133. Surface markers on DC (lower compartment) were determined by flow cytometry after 18 h incubation. Controls: untreated cells (control) and lipopolysaccharide-stimulated cells (LPS). Results were expressed as the medians of fluorescence intensity (MFI)  $\pm$  standard deviation from two independent experiments. \* indicate significant differences with control ( $P<0.05$ ).



significant differences for any of the treatments tested (Figure 5A).

Production of IL-6 showed different pattern of stimulation as compared with IL-8. Values similar to basal levels were observed when cells were stimulated with either LPS

(128.3±11.6 pg/ml) or strain CIDCA 133 (87.6±123.9 pg/ml), while a significant increase was observed when cells were incubated with strain B10502 (890.6±263 pg/ml). The presence of *Lactobacillus* in combination with *B. cereus* did not modify IL-6 production (1,091.52±55.73 pg/ml) as compared to stimulation with *B. cereus* alone (Figure 5B).



**Figure 5.** Cytokine production (A – IL-8; B – IL-6; and C – TNF- $\alpha$ ) by monocyte-derived dendritic cells co-cultured with HT-29 cells, *Lactobacillus delbrueckii* subsp *lactis* CIDCA 133 and *Bacillus cereus* B10502. Epithelial cells (upper compartment) were infected with *B. cereus* B10502 in the presence or absence of *L. delbrueckii* subsp *lactis* CIDCA 133. After 18 h incubation, cytokines were determined in the lower compartment (DC). Controls: untreated cells (basal) and lipopolysaccharide-stimulated cells (LPS). Results were expressed in pg/ml. Data are shown as the mean  $\pm$  standard deviation from three independent experiments. \* indicate significant differences ( $P<0.05$ ) with control. ▲ indicate significant differences between compared treatments ( $P<0.05$ ).

Lactobacilli and *B. cereus* in single cultures were able to increase TNF- $\alpha$  production ( $P < 0.05$ ) in co-cultures as compared to uninfected control. Of note, when both lactobacilli and *B. cereus* were added to the epithelial compartment, a significant increase in TNF- $\alpha$  production was observed ( $5,990.5 \pm 421.3$  pg/ml,  $P < 0.05$ ) as compared with cells treated with individual strains (Figure 5C). Production of IL-10 and IL-12p70 was not detectable under any of the experimental conditions of the present study (data not shown).

#### 4. Discussion

In the present study the immunomodulatory effect of a potentially probiotic strain on *B. cereus*-infected cells was demonstrated through *in vitro* studies with cultured human epithelial and DC. By using cultures of HT-29 cells, we demonstrate that infection with *B. cereus* leads to the production of IL-8 following induction of NF- $\kappa$ B pathway. Studies with co-cultures of epithelial and DC allowed us to get a further insight and show that infection of human epithelial cells induces phenotypic and functional maturation of DC and production of pro-inflammatory cytokines. Interestingly, the presence of *L. delbrueckii* subsp. *lactis* CIDCA 133 modifies cell response.

Adhesion of microorganisms to enterocytes and secretion of extracellular factors can induce IL-8 production in epithelial cells. This has been demonstrated for example for *Helicobacter pylori* (Papadakos *et al.*, 2013), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) (Edwards *et al.*, 2011), enteroinvasive *E. coli* (Jung *et al.*, 1995), *Yersinia enterocolitica* (Jung *et al.*, 1995; Schulte *et al.*, 1996), *Salmonella dublin*, *Shigella dysenteriae* and *Listeria monocytogenes* (Jung *et al.*, 1995). In contrast, potentially probiotic microorganisms belonging to genera *Bifidobacterium* and *Lactobacillus* not only do not induce IL-8 production in epithelial cells (Imaoka *et al.*, 2008; Lammers *et al.*, 2002) but also have the ability to reduce the activation of the NF- $\kappa$ B pathway in epithelial cells (Chen, 2013; Grompone *et al.*, 2012). This potentially anti-inflammatory effect was also demonstrated for yeasts isolated from kefir that were also able to decrease flagellin-mediated activation of NF- $\kappa$ B (Romanin *et al.*, 2010). Agonists involved in these effects comprise genomic DNA and cell wall components (Hiramatsu *et al.*, 2013). Lipoteichoic acid (LTA) is a key cell wall component of Gram (+) bacteria. However, even though LTA from pathogenic and non-pathogenic bacteria share many common structures, it has been demonstrated that biological responses are different. Indeed, whereas LTA from pathogens induce inflammation, those from non-pathogens can even decrease inflammation (Kim *et al.*, 2012a).

We determined that the presence of strain CIDCA 133 did not modified viability of *B. cereus* (data not shown). In contrast, in our experimental conditions, there was an effect of chloramphenicol on the viability of all the *B. cereus* strains under study. Even if this is a bacteriostatic agent that does not modified *B. cereus* viability in short experiments (Minnaard *et al.*, 2004, 2013), there was a significant reduction of *B. cereus* viability (4 log) after 18 h incubation with 100  $\mu$ g/ml chloramphenicol (data not shown). Since no bacterial lysis was observed, the number of total bacteria remained unchanged. It is important to point out that all the *B. cereus* strains under study decreased to similar values of cfu and that the presence of strain 133 did not modify these values. On the other hand, viability of strain 133 did not change in the presence of chloramphenicol in the 18 h incubation period. Therefore, the effect of strain 133 cannot be ascribed to a reduction of total *B. cereus* concentrations. The supplementation of culture media with antibiotics is mandatory in cell stimulation experiments with *B. cereus* since even short incubation periods (3 h) in antibiotic-free media lead to complete destruction of the cell monolayers due to bacterial growth. Supplementation of culture media with antibiotics is a common practice in the studies of interaction between bacteria and eukaryotic cells. Furthermore, many studies have been performed in the presence of bactericidal agents, such as penicillin and streptomycin (Carasi *et al.*, 2015; Huang *et al.*, 2015; Klingspor *et al.*, 2015; Mastropietro *et al.*, 2015). It is worth noting that it has long been demonstrated that stimulation of cell response occurs even when cells are incubated with non-viable bacteria or cellular fractions (Jeon *et al.*, 2015; Kaji *et al.*, 2010).

Probiotic lactobacilli can modulate immune responses by signalling through NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) pathways (Van Baarlen *et al.*, 2009). Of note, live *Lactobacillus rhamnosus* increases activation of NF- $\kappa$ B in human bladder cells but decreases production of TNF- $\alpha$ , IL-6 and CXCL8 (Karlsson *et al.*, 2012). In the present study, we show that activation of NF- $\kappa$ B triggered by *B. cereus* in HT-29 cells increased in the presence of *L. delbrueckii* subsp. *lactis* CIDCA 133 but with a decrease of IL-8 production. These findings emphasise the relevance of alternative transcription factors other than NF- $\kappa$ B (e.g. MAPK) and in addition the effect of post-transcriptional regulation of NF- $\kappa$ B. Furthermore, effect of extracellular factors produced by *L. delbrueckii* subsp. *lactis* CIDCA 133 cannot be ruled out (Garrote *et al.*, 2015; Iraporda *et al.*, 2015; Karlsson *et al.*, 2012; Tanaka *et al.*, 2015).

Co-cultures of epithelial and DC allow for the study of the cross talk between cell populations relevant for the host's response and pathogenic or beneficial microorganisms. Since early studies on the immune response elicited by probiotic microorganisms (Haller *et al.*, 2000) this *in vitro* model has demonstrated to be useful for the study

of the interaction of microorganisms in the context of intestinal infections (Fang *et al.*, 2010; Haller *et al.*, 2000; Pozo-Rubio *et al.*, 2011). This experimental approach allowed the demonstration of the immunomodulatory effect of *L. rhamnosus*, which decreases HLA-DR and CD86 expression marker on DC in a LPS-stimulated co-culture system (Grompone *et al.*, 2012). In contrast, other *Lactobacillus* strains induced HLA-DR and CD86 expression in DC from human origin (Mohamadzadeh *et al.*, 2005). Interestingly, other potentially probiotic strains modified the expression of MHCII, CD86 and CD40 and the production of inflammatory cytokines (IL-6 and TNF- $\alpha$ ) by murine DC depending on the presence of epithelial cells in normal and inverted co-culture systems (Kim *et al.*, 2012b).

Presence of *B. cereus* or *L. delbrueckii* subsp. *lactis* CIDCA 133 alone, lead to stimulation of CD86 in the absence of LPS. This suggests a signalling pathway other than Toll-like receptor (TLR)-4-mediated. The presence of lactobacilli does not modifies CD86 expression. It is worth to note that epithelial cells (HT-29) in the upper compartment of co-cultures, were stimulated at the apical domain and that translocation of *B. cereus* to the lower compartment was reduced by the presence of *L. delbrueckii* subsp. *lactis* CIDCA 133.

In spite that number of DC associated to *B. cereus* in the lower compartment significantly decreases when epithelial cells at the upper compartment were incubated with both lactobacilli and *B. cereus*, there were no changes in CD86 expression in DC. These findings support the hypothesis that stimulation of epithelial cells in the upper compartment generates a cross-talk with DC of the lower compartment through soluble factors.

Concerning functional maturation, determination of cytokines demonstrates that both strains were able to induce IL-8 and TNF- $\alpha$  production by DC but only *B. cereus* was able to trigger IL-6 production. Interestingly, our results showed that whereas presence of *L. delbrueckii* subsp. *lactis* CIDCA 133 did not modify the production of IL-8 nor IL-6 by *B. cereus*-infected DC, this *Lactobacillus* significantly increased TNF- $\alpha$  production. The ability of lactobacilli to trigger TNF- $\alpha$  production by macrophages through TLR-2 signalling has been demonstrated (Ditu *et al.*, 2014; Matsuguchi *et al.*, 2003). In addition, spent culture supernatants of *B. cereus* induce TNF- $\alpha$  production in holoxenic mice (Ditu *et al.*, 2014). Furthermore, in a murine model of infection (Rolny *et al.*, 2014), mice infected with *B. cereus* strain B10502 show slight increases in expression of genes associated to TNF- $\alpha$  production. In the present work, the addition of *L. delbrueckii* subsp. *lactis* CIDCA 133 potentiates TNF- $\alpha$  production by DC in the lower compartment of a co-culture system with *B. cereus*-infected epithelial cells. Although TNF- $\alpha$  is a pro-inflammatory cytokine, it has been shown to play a role in the containment

of *B. cereus* in a murine model of intraocular infection. Production of TNF- $\alpha$  at early stages of infection leads to the recruitment of immune cells that in turn contribute to the control of pathogen multiplication (Ramadan *et al.*, 2008).

Surprisingly, strain 133 was unable to induce neither IL-10 nor IL-12. Induction of these interleukins is a typical biological response following interaction between probiotics and host's cells. This strain-dependent response is related to differential activation of the ERK pathway by different bacterial components such as CpG motifs and cell wall components (Kaji *et al.*, 2010). Strong inducers of the ERK pathway lead to high levels of IL-10 and low levels of IL-12 whereas those bacteria that shows a weak ability to induce ERK pathway lead to high values of IL-12 and low values of IL-10. However, other signalling pathways are involved in the production of IL-10/IL-12 and some strains that share many common agonists (e.g. lypoteichoic acids) are unable to induce these interleukins. Interestingly, these microorganisms have shown faster intracellular digestion by macrophages (Kaji *et al.*, 2010).

The present work demonstrates for the first time the effect of *L. delbrueckii* subsp. *lactis* CIDCA 133, a potentially probiotic strain, in an *in vitro* model of *B. cereus* infection. Our results showed that the presence of the lactobacilli lead to the modulation of the cell response of infected cells and an enhancement of previously demonstrated protective response such as TNF- $\alpha$  production by DC.

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