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ORIGINAL PAPER

Lactobacillus delbrueckii subsp. *lactis* (strain CIDCA 133) stimulates murine macrophages infected with *Citrobacter rodentium*

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Abstract *Citrobacter rodentium* is a specific murine enteropathogen which causes diarrheal disease characterized by colonic hyperplasia and intestinal inflammation. Recruitment of neutrophils and macrophages constitute a key step to control the infection. Since modulation of the activity of professional phagocytic cells could contribute to improve host´s defences against *C. rodentium*, we investigated the effect of *Lactobacillus delbrueckii* subsp. *lactis* (strain CIDCA 133) on the interaction between murine macrophages (RAW 264.7) and *C. rodentium*. Phagocytosis, surface molecules and inducible nitric oxide synthase (iNOs) expression were determined by flow cytometry. Reactive oxygen species (ROS) were assessed by fluorescence microscopy. The presence of lactobacilli increased phagocytosis of *C. rodentium* whereas *C. rodentium* had no effect on lactobacilli internalization. Survival of internalized *C. rodentium* diminished when strain CIDCA 133 was present. CD-86, MHCII, iNOs expression and nitrite production were increased when *C. rodentium* and lactobacilli were present even though strain CIDCA 133 alone had no effect. Strain CIDCA 133 led to a strong induction of ROS activity which was not modified by *C. rodentium.* *Lactobacillus delbrueckii* subsp. *lactis* (strain CIDCA 133) is able to increase the activation of murine macrophages infected with *C. rodentium*. The sole presence of lactobacilli is enough to modify some stimulation markers (e.g. ROS induction) whereas other markers require the presence of both bacteria; thus, indicating a synergistic effect.

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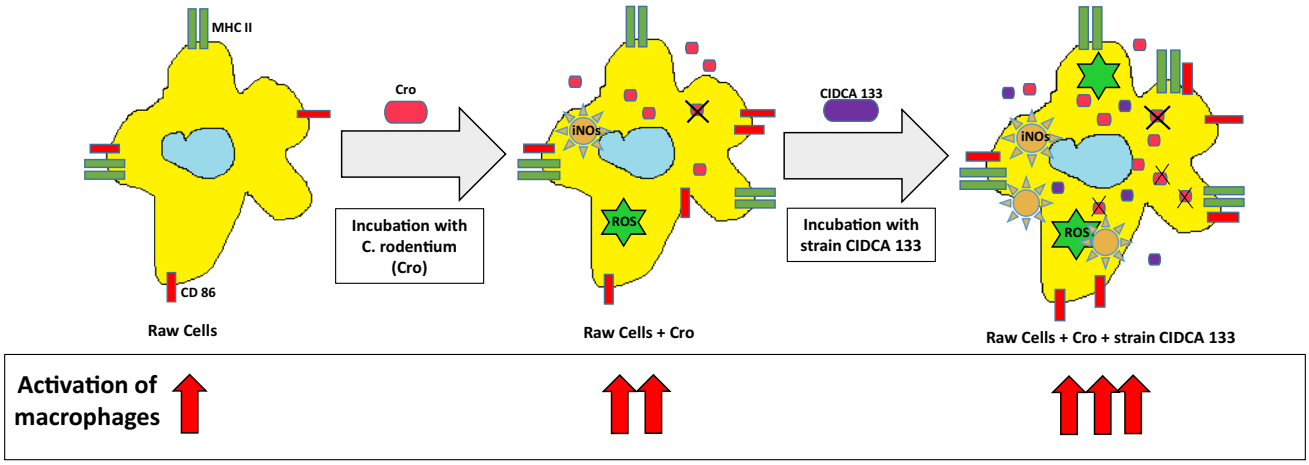
Graphical Abstract



Keywords *Citrobacter rodentium* · Lactobacilli · Macrophages · Phagocytosis · Probiotics

Introduction

Citrobacter rodentium, is the etiologic agent of transmissible murine colonic hyperplasia, a naturally occurring disease of laboratory mice characterized by epithelial cell hyperproliferation in the descending colon, crypt hyperplasia, loss of goblet cells, and mucosal infiltration with macrophages, lymphocytes, and neutrophils (Luperchio et al. 2000; Mundy et al. 2005). The infection of adult mice is generally self-limiting, with low morbidity and mortality. Infected mice generally clear *C. rodentium* from the intestine and recover in approximately 4 weeks post infection (Ghaem-Maghami et al. 2001; Luperchio and Schauer 2001; Mundy et al. 2005). However, some mice strains (e.g. FVB/N or C_3 H) and suckling mice are more susceptible, developing diarrhea, slow growth, and rectal prolapse that is associated with colonic inflammation and high mortality ratios (Vallance et al. 2003; Borenshtein et al. 2008).

Adhesion of *C. rodentium* to enterocytes leads to typical intestinal attaching-effacing (A/E) lesions. These epithelial injuries are characterized by an intimate attachment of the pathogen to the apical domain of the cells and a subsequent effacement of the microvilli. This microorganism serves as a model for human A/E pathogens such as enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) (Collins et al. 2014).

The infection of *C. rodentium* is characterized by mucosal infiltrates of CD4 (+) T cells associated with colonic hyperplasia. These infiltrates have a Th1 phenotype with an increased expression of cytokines (TNFα, IFNγ and IL-12) (Higgins et al. 1999). It has been recently demonstrated that Th-17 pathway is also involved, with an up-regulation of the production of IL-6 and IL-17 and recruitment of T_{reg} $CD25^+CD4^+FoxP3^+$ cells (Symonds et al. 2009).

Innate and adaptive responses are necessary to control *C. rodentium* infection (Simmons et al. 2003; Bry and Brenner 2004). At the first steps of infection, cell signaling mediated by Toll-like receptors (TLR) induces the production of cytokines that leads to the recruitment of neutrophils and macrophages (Lebeis et al. 2008). Neutrophils and macrophages play a key protective role at early stages of the infection since they contribute to the containment of bacteria within the colon, reducing its proliferation and dissemination (Lebeis et al. 2007; Kum et al. 2010). Neutrophils are the first recruited cells and they are capable of directly kill *C. rodentium* thus, controlling dissemination of the microorganism. Indeed, depletion of neutrophils in wild type mice resulted in an increased number of colonic bacteria as well as an increased translocation (Lebeis et al. 2007). The arrival of macrophages to the infection site contributes to eliminate *C. rodentium* and constitutes a crucial step both for the reparation of the intestinal barrier and for antigen presentation (Lebeis et al. 2008). Moreover, monocytes and macrophages shape the adaptive immune response to *C. rodentium* by promoting the development of IFN-γ-producing Th-1 cells (Schreiber et al. 2013).

Several reports confirm that the administration of probiotics could ameliorate the symptoms of *C. rodentium* infection in mice. Different lactobacilli strains showed the ability to avoid the epithelial barrier dysfunction, reduce the colonic hyperplasia, decrease the inflammation, diminish the pathogen concentration and normalize the colonic microbiota (Varcoe et al. 2003; Chen et al. 2005; Johnson-Henry et al. 2005; Gareau et al. 2010; Rodrigues et al. 2012). These protective effects are in part a consequence of the interaction between probiotics and cells of the innate immune system. In fact, adoptive transfer of dendritic

cells (DC) from lactobacilli-treated mice increased the IL-12 and INFγ productions as well as Ig A levels of mice infected with *C. rodentium* (Chen et al. 2009). In addition, different reports suggest that the stimulation of murine peritoneal macrophages with lactic acid bacteria in vitro or by oral administration leads to an increase in IL-12, ROS production and phagocytic activity (Foo et al. 2011; Ichikawa et al. 2012).

Lactobacillus delbrueckii subsp. *lactis* (strain CIDCA 133) is a potentially probiotic strain that has shown interesting properties such as the ability to inhibit harmful enzymatic activities and the capacity to antagonize pathogenic microorganisms such as enterohaemorrhagic *E. coli*, an intestinal pathogen that shares virulence mechanisms with *C. rodentium* (Hugo et al. 2006, 2008).

Considering the importance of the innate immunity to control *C. rodentium* infection and the probiotic capacity to enhance macrophage activity, we investigated the interaction between murine macrophages (RAW 264.7 cells), *C. rodentium* and a selected lactobacillus strain (CIDCA 133).

Materials and methods

Microorganisms and culture conditions

Lactobacillus delbrueckii subsp. *lactis* (strain CIDCA 133) belongs to the CIDCA culture collection and *C. rodentium* ICC 180 was kindly provided by Dr. Thomas T. Macdonald of the Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, London, United Kingdom. Lactobacilli were grown in De Man Rogosa Sharp (MRS) broth at 37°Cfor 16 h. *C. rodentium* was cultured in Luria–Bertani medium at 37 °C for 16 h with agitation. Stock cultures of both strains were stored at −80 °C. Two sub-cultures were performed before the assays.

Preparation of FITC-labeled bacteria

Bacterial cultures were centrifuged and washed with phosphate buffered saline (PBS). Pellets were suspended at a concentration of 1×10^9 CFU ml^{-1} in 0.1 mg ml^{-1} fluorescein isothiocyanate (FITC, Sigma, St. Louis, MO), 50 mM $NaHCO_3$ and 100 mM NaCl (pH 7) for 16 h at 4 °C. Bacterial cells were then washed three times with PBS and suspended in PBS at different concentrations.

Cell culture

The monocyte/macrophage murine cell line RAW 264.7 was routinely cultured in DMEM (Gibco BRL Life Technologies, Rockville, MD, USA) supplemented with: 10% (v/v) fetal bovine serum (FBS), (PAA Laboratories, GmbH, Pasching, Austria), 1% (v/v) nonessential amino acids (GIBCO BRL Life Technologies, Rockville, MD, USA), and antibiotics 1% (v/v) penicillin–streptomycin solution (100 U ml^{-1} penicillin G, 100 µg ml^{-1} streptomycin). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells in the experiments were used 48 h after seeding.

Phagocytosis assay

Cell monolayers were washed twice with PBS and then incubated in DMEM for 1 h with FITC-labeled bacteria at different multiplicities of infection (m.o.i. = ratio of bacteria per RAW 264.7 cell). Afterwards cells were washed twice with cold PBS and transferred to FACS tubes. Trypan blue quenching solution (Voyich and DeLeo 2002) was used to discriminate the fluorescence signal of bacteria adhering to the extracellular surface from those within the cells. The quenching solution was added to the samples immediately prior to the flow cytometry acquisition.

The additional determinations in this work (e.g. iNOs, surface molecules expression) were performed after the phagocytosis assay. In co-incubation experiments both bacteria were added at the same time.

Determination of bacterial association and internalization

To determine the number of associated bacteria monolayers were incubated for 1 h at 37 °C with *C. rodentium* and/or strain CIDCA 133 to allow for phagocytosis. Then, monolayers were washed three times (PBS) and they were lysed with sterile distilled water containing 0.1% of Triton X-100 for 1 h at 37 °C. Cell suspensions were mechanically disrupted by 6 passages through a syringe with a 22 G needle. The number of associated bacteria was determined by plating appropriate dilutions on nutrient agar.

To determine the number of internalized bacteria, monolayers were incubated for 1h with the bacterial suspensions, washed and further incubated for 2 h at 37 °C with gentamicin (100 µg ml^{-1}), in order to kill non-internalized bacteria. After being washed with PBS to remove the antibiotic, cells were lysed with distilled water-Triton X-100 as described above. The number of internalized bacteria was assessed by plate counts.

Expression of surface molecules

After 48 h of incubation with microorganisms, the expression of CD86 and MHCII in RAW 264.7 cells was assessed. Cells were washed twice with cold PBS and harvested by scrapping. Samples were incubated with PE-conjugated

monoclonal antibody anti-CD86 or anti-MHCII (BD, Pharmingen, CA, USA). Cell samples were washed twice and then they were analyzed by flow cytometry. Cells stimulated with LPS (1 μg ml^{-1}) (Sigma, St. Louis, MO) were used as positive control.

Inducible nitric oxide synthase (iNOs) expression

After 48 h of the phagocytosis assay, RAW 264.7 cells, at a concentration of 1×10^6 cells ml^{-1}, were fixed and permeabilized with saponin buffer (0.1% p/v saponin and 2% FBS v/v in PBS). Cells were incubated with anti-iNOS- PE monoclonal antibody (Santa Cruz, Biotechnology) diluted in saponin buffer for 60 min at room temperature. Cells were washed twice with PBS prior to flow cytometry analysis. RAW 264.7 cells stimulated with LPS (1 μg ml^{-1}) were used as positive control.

Quantification of nitrite production

Nitric oxide (NO) production was indirectly measured by determining nitrite levels. The nitrite concentration from culture supernatants obtained after 48 h of the phagocytosis assay was determined according to McNamara et al. (1971). Equal volumes of cell culture supernatants, 1% (w/v) sulphanilic acid and 0.02% (w/v) *N*-naphthyl-ethylene-diamonium dichloride (both in 1.5 M HCl) were mixed. The mixtures were allowed to stand for 10 min at room temperature for color development and optical density at 540 nm was measured in a Synergy HT fluorescence microplate reader (Bio-Tek Instruments, Winoski, VT, USA). Sodium nitrite was used as standard. The nitrite production was expressed as the ratio between nitrite and cell concentration. Cell concentration was determined in a haemocytometer.

Detection of reactive oxygen species production (ROS)

ROS production was determined by fluorescence microscopy by using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate acetyl ester (CM-H2DCFDA) (Invitrogen, CA, USA). RAW 264.7 cells were incubated for 30 min with *L. delbrueckii* subsp. *lactis* (strain CIDCA 133), *C. rodentium* ICC 180 or a combination of both microorganisms. Hydrogen peroxide (50 mM) and Hanks buffered saline solution (HBSS) were used as positive or negative controls respectively. Afterwards, cells were washed and incubated in the dark with 3.5 μM of CM-H2DCFDA for 15 min and washed in HBSS. Microscopy was performed by means of a Nikon Eclipse Ti-U microscope (Nikon, Tokyo, Japan) and fluorescence was quantified using an automated macro for ImageJ which analyzes the mean fluorescence intensity in the image (Rasband 1997; Abràmoff et al. 2004). Fluorescence intensity values were referred to the basal condition without bacteria and expressed as fluorescence fold increase (FFI).

Flow cytometry analysis

Flow cytometry analysis was performed in a FACSCalibur flow cytometer (FACSCalibur, CellQuest software; Becton Dickinson, Mountain View, CA, USA). FITC fluorescence was measured in the FL1 channel (515–545 nm) and the PE fluorescence was measured in the FL2 channel (564–601 nm). The ratio of FITC (+) or PE (+) cells was determined from FL1/2-H vs FSC-H scatter plots. For each sample 10.000 events were acquired.

Statistical analysis

Results represent the means from at least three independent experiments ± standard deviation of the mean. Analysis of variance (ANOVA) was carried out by using the statistical program Statistix 8 Software (Analytical Software, Florida USA). Means were compared by using the Tukey's test.

Results

Phagocytosis assays

The ability of the macrophage cell line RAW 264.7 to internalize FITC-labelled *C. rodentium* in non-opsonic conditions was dependent on the m.o.i. Indeed, ratios of FITC (+) cells ranging from 19 to 96% were obtained for m.o.i. between 25:1 and 100:1, respectively (Fig. 1a). Interestingly, when *L. delbrueckii* subsp. *lactis* strain CIDCA 133 was present, the percentage of FITC positive cells significantly increased ($p < 0.05$) for the m.o.i. 50:1 and 25:1 (Fig. 1a). Histograms of FL-1 positive cells clearly showed the displacement of the curves to higher values when strain CIDCA 133 was present in m.o.i. 50:1 and 25:1 (Fig. 1b). Phagocytosis of *C. rodentium* at the highest m.o.i. (100:1) was not modified by lactobacillus, due to the high percentage of internalization of *C. rodentium* in this condition (Fig. 1a, c). These findings indicate that there was an increase in the ratio of phagocytic cells with intracellular *C. rodentium* in co-incubation experiments with strain CIDCA 133. In contrast, when FITC-labelled strain CIDCA 133 was incubated with phagocytic cells the internalization of lactobacilli was not modified by the presence of *C. rodentium* (Fig. 1c). On the basis of the above results, we chose a m.o.i. of 25:1 of *C. rodentium* and 20:1 of strain CIDCA 133 for further experiments.

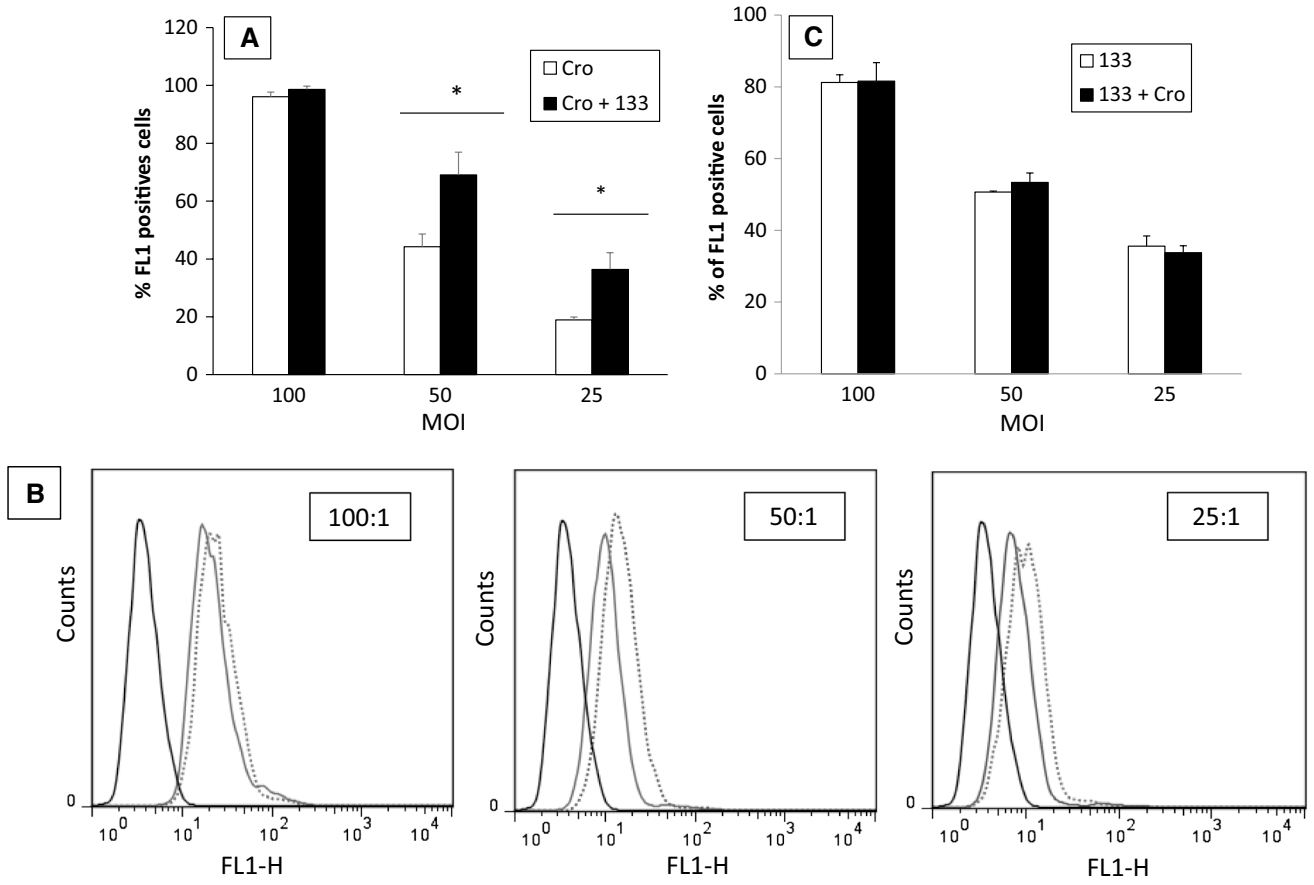


Fig. 1 Interaction of FITC-labeled bacteria with RAW 264.7 cells after 1 h incubation. Non internalized bacteria were quenched with trypan blue. **a** Percentage of FITC (+) cells after incubation with FITC-labeled *C. rodentium* (Cro) at different multiplicity of infection (m.o.i.). *White bars* corresponds to cells incubated with *C. rodentium* alone. *Black bars* corresponds to cells incubated with *C. rodentium* and strain CIDCA 133 at m.o.i. 25. **b** Histograms of FITC (+) cells after incubation with FITC-labeled *C. rodentium* at different m.o.i. *Black lines* indicates control cells, *gray solid line* indicates cells incubated with *C. rodentium; gray dotted line* indicates cells incubated with *C. rodentium* and strain CIDCA 133. **c** Percentage of FITC (+) cells after incubation with FITC-labeled strain CIDCA 133 at different m.o.i.; without (*white bars*) or with *C. rodentium* at m.o.i. 25:1 (*black bars*). *Asterisks* indicate significant differences ($p < 0.05$)

Table 1 Viable counts of *C. rodentium* associated to or internalized in RAW 264.7 cells

	Associated bacteria	Internalized bacteria
Cro	$1.17 \times 10^6 \pm 1.00 \times 10^5$ [a]	$7.00 \times 10^4 \pm 7.94 \times 10^3$ [b]
Cro + 133	$1.12 \times 10^6 \pm 1.36 \times 10^5$ [a]	$5.13 \times 10^4 \pm 1.53 \times 10^3$ [c]

Values are expressed in CFU ml^{-1}. Different letters express significant differences at $p < 0.05$

Viability of phagocytosed bacteria

Adhesion to RAW 264.7 cells and survival of internalized *C. rodentium* were evaluated by viable counts (Table 1). Association of *C. rodentium* to RAW 264.7 cells was independent of the presence of lactobacilli; however, survival of internalized *C. rodentium* was significantly lower when strain CIDCA 133 was present. Indeed, viability of phagocytosed *C. rodentium* in co-incubation experiments decreased approximately by 30%.

Determination of surface molecules

Given that internalization of microorganisms greatly influences activation and differentiation of phagocytic cells, we analyzed the expression of the co-stimulatory marker CD86 as well as MHCII in RAW 264.7 cells.

Infection with *C. rodentium* significantly increased the percentage of CD86 and MHCII positive cells (Fig. 2). In contrast, cells that were incubated only with strain CIDCA 133 expressed basal levels of both markers (Fig. 2). It is noteworthy that when *C. rodentium* and strain CIDCA 133 were assayed together, there was a significant increase in the expression of both surface molecules. In fact, values of MHCII and CD86 positive cells were 1.6 and 1.3 fold

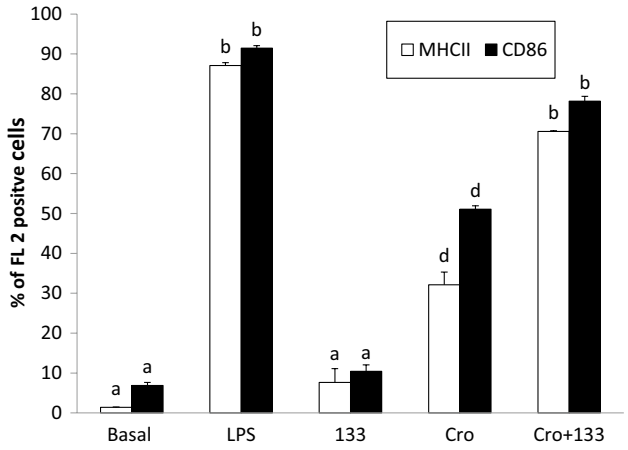


Fig. 2 Expression of surface markers in RAW 264.7 cells after 48 h infection with *C. rodentium* at m.o.i. 25. *White bars* represent the percentage of MHCII (+) cells. *Black bars* represent the percentage of CD86 (+) cells. When present, m.o.i. for strain CIDCA 133 was 25. *Different letters* indicate significant differences ($p < 0.05$)

higher respectively than those for cells incubated only with *C. rodentium* at 48 h (Fig. 2).

Effect on reactive oxygen and nitrogen species

A significant increase in the ratio of iNOs (+) cells was found after incubation of cells with *C. rodentium* for 48 h (Fig. 3a). In contrast, single suspensions of lactobacilli did not induce changes in the ratio of iNOs (+) cells. Interestingly, the presence of both strains increased the ratio of iNOs (+) cells even more, reaching percentages similar to LPS (Fig. 3a).

In order to assess the production of nitrogen reactive species, we evaluated the concentration of nitrite as an indirect measure of NO production. As shown in Fig. 3b, nitrite production by *C. rodentium*-infected cells was significantly increased when strain CIDCA 133 was also present. Interestingly, the sole presence of lactobacilli did not increase nitrite production as compared with basal values in unstimulated controls.

To gain further insight into the stimulation of the microbicidal activity of macrophages, we evaluated the production of reactive oxygen species (ROS) in infected cells. ROS values in cells incubated with *C. rodentium* were similar to those of unstimulated controls, whereas there was an induction of ROS when cells were stimulated with both microorganisms. This increase correlated with the presence of strain CIDCA 133 (Fig. 4). Strain CIDCA 133 was negative for ROS production (data not shown); therefore, ROS activity in the experiments corresponds exclusively to eukaryotic cells.

Discussion

It is known that viable probiotic bacteria or their cellular components (e.g. cell wall components) induce the production of pro-inflammatory cytokines and enhance phagocytosis and nitric oxide production by macrophages in vitro (Tejada-Simon and Pestka 1999; Korhonen et al. 2001; Kim et al. 2007). Several reports demonstrated that the ingestion of lactobacilli activates peritoneal macrophages in mice and rats (Foo et al. 2011; Yoda et al. 2014). Also in humans, (Schiffrin et al. 1997; Gill et al. 2001) an effect on the phagocytic activity has been demonstrated by oral consumption of lactobacilli. Moreover,

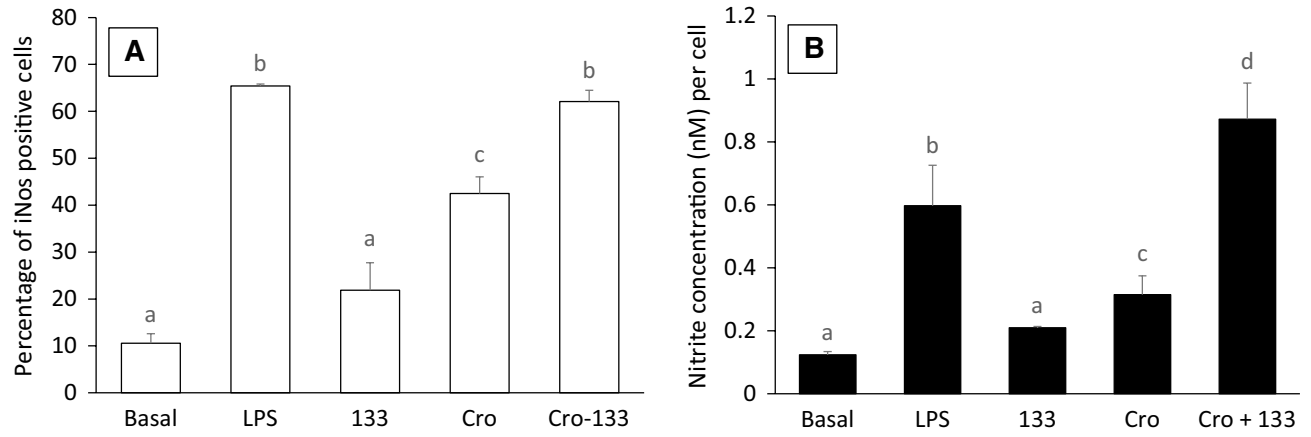


Fig. 3 Determination of markers related to reactive nitrogen species (RNS) in RAW 264.7 cells after 48 h infection with *C. rodentium* at m.o.i. 25. **a** Percentage of iNOs positive cells. **b** Nitrite production. When present, strain CIDCA 133 was at m.o.i. 25. *Different letters* indicate significant differences ($p < 0.05$)

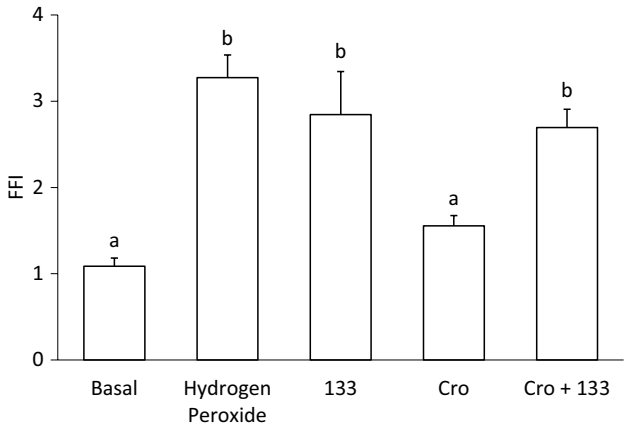


Fig. 4 Production of reactive oxygen species (ROS) by RAW 264.7 cells. Fluorescence measures were referred to basal values without bacteria and expressed as fluorescence fold increase (FFI). Positive control was prepared by adding 50 mM hydrogen peroxide. *Different letters* indicate significant differences ($p < 0.05$). *C. rodentium* and strain CIDCA 133 were at m.o.i. 25

the stimulation of macrophages by oral administration of probiotics could be detected in non-intestinal localizations (Kotzamanidis et al. 2010; Marranzino et al. 2012).

Citrobacter rodentium is a murine pathogen that causes a colonic infection which could be life threatening for some mice strains and suckling mice. Since interaction of *C. rodentium* with intestinal epithelium lead to attaching-effacing (A/E) lesions, this microorganism constitutes an accepted model for A/E human pathogens such as enterohaemorrhagic *E. coli* (EHEC). Neutrophils and macrophages play a key role in control *C. rodentium* infection. In this work, we studied the interaction between a potentially probiotic microorganisms *L. delbrueckii* subsp. *lactis* strain CIDCA 133 with cultured murine macrophages (RAW 264.7 cells) infected with *C. rodentium*.

Kinetic studies revealed that phagocytic cells start internalization of bacteria in a short period of time. Indeed there are intracellular bacteria 5 min after infection and there is a time-dependent increase reaching a plateau between 30 and 60 min (Lu et al. 2014). In the present paper, we evaluated phagocytosis after 30 min (not shown) and 60 min incubation. We found that at 60 min incubation there is a significant biological effect.

Concerning killing of intracellular bacteria, we evaluated the survival of internalized *C. rodentium* after 3 and 16 h (not shown) of incubation. Viability of internalized *C. rodentium* after 16 h of incubation decreased 1.5 log as compared with viability after 3 h of incubation. Incubation of *C. rodentium* and strain CIDCA 133 in the same conditions of the phagocytosis experiments but without phagocytic cells revealed that the decrease in viability is not due to any direct bactericidal activity of strain CIDCA 133 (data not shown).

Our results show that, there is an increase of phagocytosis of *C. rodentium* and a diminution in survival of the internalized enteropathogen in the presence of strain CIDCA 133. The increase of phagocytic and killing capacity of macrophages constitutes an important finding considering that *C. rodentium* could interferes with phagocytic activity by injection of the effector factor EspH into host cells through type III secretion system (TTSS) (Dong et al. 2010).

Different reports described the ability of lactobacilli to stimulate the expression of surface markers (TLRs, CDs, MHCII, etc.) in macrophages and DC (Chen et al. 2009; Wang et al. 2013). Even though strain CIDCA 133 alone did not induces significant expression of MHCII nor CD86, this strain can increase the stimulation triggered by *C. rodentium*.

The ability to induce iNOs expression and NO production varies among lactobacilli strains (Jeong et al. 2015). Even though several probiotic strains induce NO production in macrophages per se (Tejada-Simon and Pestka 1999; Kim et al. 2007; Wang et al. 2013), this effect was not found in strain CIDCA 133. Interestingly, the co-incubation of strain CIDCA 133 and *C. rodentium* significantly, enhances nitrite production of the infected cells.

It has been demonstrated that, different species of lactobacilli induce generation of ROS in cultured cells, mice and *Drosophila* larvae whereas gramnegative microorganisms lead to less stimulation (Jones et al. 2013). In addition, there is a correlation between ROS production, IL-12 secretion and phagocytosis in murine macrophages stimulated with lactobacilli strains (Ichikawa et al. 2012). In the present work, we show a strong induction of ROS activity in RAW 264.7 cells due to the presence of the strain CIDCA 133. The increase in ROS and NO production in RAW 264.7 cells stimulated by lactobacilli might also increase their antimicrobial capacity. It this sense, we observed that the presence of strain CIDCA 133 correlates with the diminution of viability of internalized *C. rodentium*.

It has been demonstrated that, nitric oxide (NO) has antimicrobial activity against *C. rodentium* and during infection, there is an increased expression of inducible NO synthase (iNOS) in gut epithelial cells (Vallance et al. 2002a) and increased nitrate/nitrite concentrations in serum (Simmons et al. 2003). Even though, iNOS−/− knockout mice clear infection normally (Simmons et al. 2002), there is an increase in bacterial load at late stages of infection (Vallance et al. 2002b).

Concerning ROS, they play an essential role in controlling *C. rodentium* disease. It has been shown that Rip2−/− mice, which have an overexpression of ROS during *C rodentium* infection, exhibit increased NLRP3

inflammasome activation that, in turn, are relevant in controlling *C. rodentium* (Liu et al. 2012). Moreover it has been recently demonstrated that H_2O_2 acts as a potent, negative regulator of the LEE pathogenicity island that is crucial for colonization and disease progression of *C. rodentium* and related pathogens such as EPEC and EHEC. Downregulation of virulence factors in *C. rodentium*, decreases bacterial concentration and colon hyperplasia during the infection (Pircalabioru et al. 2016).

Our results revealed that *C. rodentium* per se is not a good inducer of ROS activity in macrophages. Similar results were found by Lupfer et al. (2014). Interestingly, we demonstrate that strain CIDCA 133 enhances ROS production in RAW 297.3 cells probably promoting all the beneficial effects caused by ROS-derived metabolites. It is important to highlight, that in the present paper, ROS have been determined by using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA). This probe, reacts with several ROS and ROS-derived metabolites such as hydrogen peroxide, hydroxyl radicals and peroxynitrite (Ameizane-El-Hassani and Dupuy 2013). Therefore, ROS levels reported in the present work actually include all the above mentioned molecules.

Macrophage functions i.e. phagocytosis, antigen presentation, cytokine production and antimicrobial activity are especially relevant in both innate and adaptive immune response (Davidson et al. 1998). Classical macrophage activation (M1) is characterized by high capacity of antigen presentation as well as high interleukin-12 (IL-12) and IL-23 production. In addition, typical M1 macrophages have shown a high production of nitrogen (RNS) and oxygen (ROS) reactive species. In contrast, M2 phenotype is characterized by low activation of ROS and RNS (Mantovani et al. 2004).

The present work reveals the ability of *Lactobacillus delbrueckii* subsp. *lactis* (strain CIDCA 133) to stimulate murine macrophages infected with *C. rodentium* in vitro. Phagocytosis, production of antimicrobial species and expression of surface markers related to antigen presentation were increased in the presence of strain CIDCA 133. We can argue that strain CIDCA 133 probably shifts *C. rodentium*-infected macrophages to a M1 phenotype which is essential to control *C. rodentium* disease (Aychek et al. 2015). Our hypothesis is supported by the fact that some of the typical M1 phenotype markers were found in *C. rodentium* infected cells in the presence of strain CIDCA 133 (i.e. increase in MHCII, ROS and iNOs). However, it should be emphasized that phenotypes M1 and M2 are the extremes of a continuum that can be modulated by host–microbe interactions.

Even though TLR 2, 4 and 9 do not seem to be crucial for lactobacilli internalization (Ichikawa et al. 2012; Boye et al. 2016), TLR-mediated signaling plays a main role in modulating phagocytosis. Indeed, it has been recently demonstrated, that stimulation with TLR2 and TLR4 ligands (Pam2/3, LPS) from the plasma membrane significantly enhances *L. acidophilus* internalization. In addition, stimulation of dendritic cells with whole lactobacilli, leads to an increase in the internalization of latex beads (abiotic particles) via the activation of TLRs from the outside of the cells. Interestingly, stimulation with TLR ligands lead to different cell response as compared with stimulation of whole bacteria. These findings are related to the intracellular processing of phagocytosed bacteria that triggers different signaling pathways by recruiting receptors to intracellular compartments (Boye et al. 2016).

Further research will be necessary to completely unravel the effect of the presence of lactobacilli or, eventually, other non-pathogenic microorganisms on the course of *C. rodentium* infection.

Most of the studies that investigate the stimulation capacity of lactobacilli strains to phagocytic cells are focused on the interaction between probiotic and host cell. However, in this work, the enhancement of some cell responses (ie, surface markers, iNOs activity) were only observed among the interaction of *C. rodentium*, strain CIDCA 133 and RAW 264.7 cells. Therefore, our findings emphasize the importance of studying the effect of probiotics in the presence of specific pathogens in order to mimic the actual interactions with relevant immune cells. The results suggest a fine tuning of the cell response triggered by probiotic lactobacilli. This response is not simply the sum of parts but encompasses an evident synergistic component that depends on the specific pathogen under study.

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