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Lactobacillus reuteri CRL1101 beneficially modulate lipopolysaccharide-mediated inflammatory response in a mouse model of endotoxic shock

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ABSTRACT

Lactobacillus reuteri CRL1101 was found to reduce inflammatory and oxidative damage during endotoxic shock induced by lipopolysaccharide (LPS) administration in mice. This beneficial effect was related to the capacity of both the cell structure as well as their secreted products to act synergistically to induce a tolerogenic profile in immune cells. The study supports the idea that, although probiotic microorganisms may exert their beneficial effects due to their well-known ability to modulate intestinal immunity, these effects are not restricted to the gastrointestinal tract. In addition, we demonstrated that the *in vitro* screening of lactobacilli strains is still a powerful approach to discriminate the potential probiotic bacteria for specific functions that could be later studied in animal models. From the systematic studies reported here, *L. reuteri* CRL1101, a strain with technological potential usefulness in dairy products, is proposed as novel probiotic candidate for the prevention and control of systemic inflammatory disorders.

1. Introduction

Lactic acid bacteria (LAB) are normal habitants of the gastrointestinal tract in humans and animals (Shu et al., 1999). Several LAB strains are considered beneficial to the host and as such have been used as probiotics and included in several functional foods. Modulation of host immunity is one of the most commonly alleged benefits of the consumption of probiotics (Koninkx, Tooten, & Malago, 2010; Lai et al., 2011; Lebeer, Claes, & Vanderleyden, 2012; Villena, Oliveira, Ferreira, Salva, & Alvarez, 2011). These immunobiotic microorganisms may interact with the cells of the mucosal innate immune system and regulate their responses to both pro-inflammatory and anti-inflammatory directions (Chiu et al., 2013; Haller et al., 2000; Lai et al., 2011). The conserved molecular structures of these microorganisms (microorganism-associated molecular patterns, MAMPs) may bind to pattern-recognition receptors (PRRs) expressed on immune cells and thus trigger a cascade of immunological mechanisms, such as the regulation of receptor expression or the production of cytokines (Corthesy, Gaskins, & Mercenier, 2007; Lee et al., 2013). In fact, the *in vitro* cytokine profile released to culture supernatants has been used to select and classify probiotic strains according to their immune properties (Cross, Ganner, Teilab, & Fray, 2004; Marranzino, Villena, Salva, & Alvarez 2012).

The anti-inflammatory features of immunobiotic LAB have been widely investigated (Chiu et al., 2013; Foligne et al., 2007; Lee et al., 2013; Villena, et al., 2012a). Although intestinal homeostasis has been the classic target of immunobiotics, different studies have proposed that the potential beneficial

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effects of these bacteria are not restricted to the intestine. It has been shown that both the systemic and the distal mucosal immune systems can be modulated by orally administered probiotics (Arribas et al., 2009; Baharav, Mor, Halpern, & Weinberger, 2004; Takata et al., 2011; Villena et al., 2012b). However, not all candidate immunobiotics have been proven equally efficient due to their differences in survival, persistence in the gastro-intestinal tract and specific interactions with the host immune system (Hessle, Andersson, & Wold, 2000; Maassen et al., 2000; Salva, Villena, & Alvarez, 2010; Villena et al., 2012b). Therefore, the successful selection of immunobiotic strains for specific applications may rely on the appropriate screening of a number of candidates chosen for their technological, *in vitro* and *in vivo* immune-modulatory performances (Kekkonen et al., 2008; Monteagudo-Mera et al., 2012; Salva et al., 2010).

The current demand for new products enriched with probiotics generates the need to select and characterize new strains with specific beneficial properties. In case of systemic uncontrolled inflammatory responses such as endotoxic shock and sepsis, the lack of effective treatments continue triggering elevated mortality rates in critical patients suffering these conditions (Brandenburg, Andrä, Garidel, & Gutsmann, 2011). In this regard, some few works have investigated the effects of probiotics in the lipopolysaccharide (LPS)-induced septic shock in mice, in order to establish whether, in addition to their intestinal anti-inflammatory activities, they show beneficial effects when an alteration in the systemic immune response occurs. The beneficial effects of probiotic compounds on colonic barrier function and the protection of liver in a mouse model of sepsis were demonstrated by Ewaschuk et al. (2007). The authors revealed that a pretreatment with oral probiotics could prevent the breakdown in intestinal barrier function, bacterial translocation, and hepatic damage in IL-10 deficient mice with sepsis induced by co-injection of D-galactosamine and LPS. In addition, Arribas et al. (2009) clearly reveal that mice treated with the probiotic *Escherichia coli* Nissle 1917 noticeably improved the altered immune response and decreased the production of inflammatory mediators associated with endotoxic shock induced by LPS in mice. Probiotic treatment of LPS mice resulted in a significantly increased production of IL-10 which contributed to the down-regulation of pro-inflammatory cytokines, including TNF-α. Other study showed that intraperitoneal injection of the lipoteichoic acid from *Lactobacillus plantarum* suppressed fatality and decreased the level of blood TNF-α in LPS-induced endotoxin shock mice (Kim et al., 2008). Although these studies show promising results, further studies are needed to find strains of technological interest with the ability to modulate the systemic inflammation and prevent its associated damage. In addition, more detailed studies are needed to gain insight in the mechanism of the systemic immunomodulatory effects of orally administered probiotics.

The aim of the present study was to select, among LAB with technological interest, those strains with potential anti-inflammatory properties and with the capacity to beneficially modulate the generalized inflammation induced by LPS administration. In addition, we aimed to evaluate if soluble factors released by LAB were involved in the anti-inflammatory effect.

2. Materials and methods

2.1. Bacterial cultures and conditioned media obtaining

Bacteria strains were provided by the Culture Collection of the Reference Centre for Lactobacilli (CERELA, Tucumán, Argentina). *Lactobacillus acidophilus* CRL1014 (isolated from human infant feces), *Lactobacillus rhamnosus* CRL1505 (from goat milk), *Lactobacillus casei* CRL75 (from a regional cheese), *Lactobacillus reuteri* CRL1101 (from human adult intestine) were grown in MRS (de Man, Rogosa and Sharpe medium, Britania, Buenos Aires, Argentina) broth at 37 °C. *Streptococcus thermophilus* CRL1190 were grown in LAPTg broth (peptone, 1.5%; tryptone, 1.0%; yeast extract, 1.0%; glucose, 1.0% w/v, and tween 80, 0.1% v/v), at 37 °C for 3 h. The microorganisms viability was determined by colony-forming unit (CFU) on MRS agar after 48 h incubation at 37 °C. Bacteria were collected by centrifugation (10 min, 8000*g*), washed twice with phosphate-buffered saline (PBS) and RPMI 1640 medium (GIBCO, Grand Island, NY, USA), and finally suspended in this medium at a concentration of 4×10^7 CFU/ml. Bacteria were incubated 4 h at 37 °C, in non-agitating conditions. LAB conditioned media (CM) was obtained by culture centrifugation; bacteria-free supernatant was aseptically filtered using 0.22 μm pore size low protein binding cellulose acetate filters (Millipore, Bedford, MA, USA) as described previously (Mechoud et al., 2012a). The cell-free supernatant (pH 7.2) was used as recovered from the filtrates or diluted as described below, for assays with PBMC or RAW 264.7 cells.

2.2. Isolation of PBMC and bioassays

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood from healthy volunteers (range 24–40 years old) by density gradient centrifugation with Ficoll-Hypaque (Histopaque-1077 Hybri-Max, Sigma, St. Louis, MO, USA). PBMC were collected from the interface, washed twice with PBS and then with RPMI 1640 medium, and suspended in this medium to a final concentration of 4×10^6/ml. Cells viability was determined by Trypan Blue (Sigma–Aldrich, St. Louis, MO, USA) dye exclusion assay. For co-culture assays PBMCs were seeded in 24-well polystyrene plates and co-incubated with viable LAB (8×10^7 UFC/ml, bacteria-cells ratio of 20:1) or CM and 0.5 μg/ml of LPS from *E. coli* O26:B6 (Sigma, St. Louis, MO, USA) at 37 °C in a humidified atmosphere of 5% CO_2, for 4, 8 and 24 h. The cultures supernatant was collected for cytokines determination.

2.3. Macrophage cultures and bioassays

Mouse macrophage cell line RAW 264.7 was used. 1×10^6/ml cells in RPMI 1640 medium were seeded in 6-well or 24-well culture plates, lowed to adhere for 8 h at 37 °C in a humidified atmosphere of 5% CO_2 prior to the addition of *L. reuteri* CRL1101 (bacteria-cells ratio of 20:1) or its CM (at a sub-cytotoxic dilution in RPMI 1640 medium) and incubated for 18 h. Then, cultures were exposed to LPS from *E. coli* serotype O26: *B6* at a final concentration of 100 ng/ml for 16 h. Cell viability was assessed by the Trypan-blue assay. The cultures

supernatant was collected for cytokines and nitric oxide (NO) determination.

2.4. Nitric oxide determination

NO production was assayed by measuring nitrite in supernatants of RAW 264.7 cells-bacteria co-cultures using the Griess reagent according to manufacturer's instructions (Promega Corporation, Madison, WI, USA).

2.5. Extracellular cytokine assay

Cytokines concentration was measured in cell free supernatants of PBMC and RAW 264.7 cells-bacteria co-cultures using enzyme-linked immunosorbent assay kits (ELISA Ready-Set-Go!, eBioscience, San Diego, CA, USA), including human and mouse TNF-α and IL-10, and mouse IL-6 and IL-1ß.

2.6. In vivo study

Male BALB/c mice, 8 weeks old, were provided by the Laboratory Animal Service of CERELA. All animal experiments described herein were carried out in accordance with protocols approved by CERELA Ethic Committee and were performed in accordance with the NIH recommendations. Mice were kept at a constant temperature of 22 ± 2 °C with 12 h light–dark cycles and provided with free access to drinking and standard rodent chow. The mice were randomly assigned to four groups ($n = 6$); two of them, designed as PBS and LPS groups, received 15% skim milk in water and the other two, named as CRL1101 and CRL1101 + LPS groups, received *L. reuteri* CRL1101 suspended in 15% skim milk in drinking water at the concentration of 2×10^8 CFU/ml daily prepared, dose that was selected in preliminary experiments (data not shown). Food and drinking intake was recorded daily. At 7 days after starting the oral administration of the strain, endotoxic shock was induced in mice from LPS and LPS + LAB groups with an intraperitoneal (i.p.) injection of 200 μl of LPS from *E. coli* O55:B5 (Sigma–Aldrich, St. Louis, MO, USA) 20 mg/kg in PBS. PBS and LAB groups received an i.p. injection of sterile PBS. This dose of LPS did not induce mice death in the next 24 h. Spleen, lung, liver and blood samples were taken on 24 h after LPS challenge as previously described (Salva et al., 2010; Villena et al., 2012b). Spleens were used to obtain splenocytes. Lungs and liver were weighed and frozen at −80 °C for myeloperoxidase (MPO) activity and oxidative stress measurements (thiobarbituric acid reactive substances and acid soluble sulfhydril groups content).

Serum TNF-α, IL-6, IL-10 and IL-1ß levels were assayed by ELISA, as described above. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, and creatinine were measured using commercially available clinical assay kits, according to the manufacturer's instructions (Wiener Lab., Rosario, Argentina).

2.7. Isolation of splenocytes and flow cytometry staining

Spleens were aseptically transfer to a tube with 3 ml RPMI 1640 medium (GIBCO) plus streptomycin (100 μg/ml) and penicillin (100 U/ml) (GIBCO). Spleens were mashed with a pipette and the splenocytes were released. Suspensions were filtered and red blood cells were lysed with 1× lysing buffer containing ammonium chloride (BD bioscience) at 4 °C for 3 min. Cells were re-suspended in RPMI medium, counted using a hemacytometer and adjusted to 2×10^6 cells by tube for staining with specific antibodies against surface antigen: FITC hamster anti-mouse CD3e, PE rat anti-mouse CD4, PE rat anti-mouse CD8a and against intracellular cytokines APC rat anti-mouse INF-γ and APC rat anti-mouse TNF (BD bioscience) as describe above. 10^5 events were acquired per tube and analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

2.8. Determination of MPO activity in lungs

MPO activity in lung was performed as described previously (Racedo et al., 2006). Briefly, lungs were washed with sterile PBS, weighted and frozen until procedures. Tissue was homogenized in 2 ml of 0.5% hexadecyltrimethyl ammonium bromide (Sigma, St. Louis, MO, USA) in 50 mM potassium phosphate buffer (pH 6.0). The homogenate was frozen at −20 °C for 15 min, thaw at room temperature, sonicated for 60 s on ice and centrifuged for 5 min. Then, 10 μl of the supernatant were mixed with 200 μl of reaction solution (16.7 mg o-dianisidine in 90 ml distilled water, 10 ml 50 mM potassium phosphate buffer, and 50 μl 1% H_2O_2). Absorbance change was recorded during 5 min at 450 nm using a microplate reader (VERSAmax, Molecular devices, Sunnyvale, CA, USA). Data were normalized using the protein amount measured by the Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA).

2.9. Lipid peroxidation measurement

Thiobarbituric acid reactive substances (TBARS) were determined in liver homogenates as the measure of oxidative damage, according to the method described by Ohkawa, Ohishi, and Yagi (1979) Briefly, liver tissue was homogenized in 4 ml of cold PBS and centrifuged at 8400*g* for 10 min. Then, 400 μl of supernatant were mixed with 200 μl of the reaction solution containing 1.5 ml of 20% acetic acid (v/v), 300 μl of 4% butylated hydroxytoluene in ethanol (w/v), 1.5 ml of 0.8% thiobarbituric acid (TBA, w/v), 300 μl of distilled water and 8% SDS. The samples were incubated 30 min in boiling water and after that 1 ml of distilled water and 2 ml of *n*-buthanol-pyridine (15:1) were added. Samples were centrifuged at 84,000*g* for 10 min. The absorbance of the supernatant was read at 532 nm with a microplate reader (VERSAmax).

2.10. Determination of non-protein sulfhydryl groups

Livers were homogenized as described above. For deproteinization, 1 ml of homogenate was mixed with 2 ml of 5% 5-sulphosalicylic acid (w/v) and centrifuged at 84,000*g* for 10 min. 40 μl of samples or standards were dispensed in a microplate, mixed with 140 μl of 143 mM buffer and 20 μl of 6 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in Na_2HPO_4 + EDTA buffer. The reaction merge were incubated at 30 °C for 5 min before the absorbance was read at 412 nm with a microplate reader (VERSAmax). The non-protein sulfhydryl groups were calculated based on a standard curve using reduced glutathione (Sigma, St. Louis, MO, USA).

2.11. Histological examination

Lung, liver and kidney samples from different groups were excised and washed out with PBS 0.01 mol/l pH 7.2. Then, tissues were immersed in 4% (v/v) formalin saline solution. Once fixed, samples were dehydrated and embedded in Histowax (Leica Microsystems Nussloch GmbH, Nussloch, Germany) at 56 °C. Finally, organs were cut into 4 μm serial sections and stained with hematoxilin–eosin for light microscopy examination. The pathologist doing the lung histology was blinded to the group of animals and the assessment of tissue sections was also blinded.

2.12. Statistical analysis

All assays were performed at least in triplicate and the results were expressed as mean values with standard deviations. Statistical analyses were performed using MINITAB 14 software (State College, PA, USA). Comparisons were accomplished by one way ANOVA general linear model. A P value <0.05 was considered statistically significant.

3. Results and discussion

3.1. Selection of lactobacilli able to modulate the production of TNF-α and IL-10 in PBMC

In the present study, we first aimed to select some potential anti-inflammatory probiotics *in vitro*. Several studies have shown that the determination of cytokines production by immune cells is useful in the characterization of a probiotic strain. Moreover, the analyses of the cytokines profiles induced by certain probiotic LAB in immune cells revealed that the most remarkable effect was the modulation of the levels of TNF-α, IFN-γ and the regulatory cytokine IL-10 for all the probiotic strains assayed (Marranzino et al., 2012; Mechoud et al., 2012a; Villena et al., 2012b). Therefore, five strains of LAB, previously selected by their technological interest (Mechoud, Juarez, de Valdez, & Rodriguez, 2012b; Ortiz, Fornaguera, Raya, & Mozzi, 2012; Rodríguez et al., 2008; Salva et al., 2010), were evaluated according to their capacities to modulate the production of TNF-α and IL-10 in PBMC. We focused our experiments first on live bacteria. After incubation of PBMC with the different lactobacilli strains a significantly release of the anti-inflammatory cytokine IL-10 was observed, specially for the strain CRL1101, that was the one with the highest values of IL-10 when compared to controls (Table 1). We also evaluated the capacity of the five strains to modulate the production of TNF-α and IL-10 in PBMC after the challenge with LPS. Stimulation of PBMC with LPS significantly increased the production of both cytokines TNF-α and IL-10. Treatment with lactobacilli increased the production of TNF-α with the exception of CRL75 which slightly reduced its levels (Table 1). In addition, *L. reuteri* CRL1101 significantly improved the production of IL-10 while the other lactobacilli reduced the levels of this cytokine (Table 1). Growing evidence suggests that the immunomodulatory properties of probiotics are strain dependent. Our current results are consistent with this general consideration of probiotics since each strain evaluated induced a different cytokine profile in PBMC. Of the strains evaluated here, *L. reuteri* CRL1101 strongly regulated the inflammatory response triggered by LPS in PBMC.

It was suggested that the final outcome of a host cell response against immunobiotic lactobacilli depends on the combination of the distinct MAMPs that can interact with the various PRRs and associated co-receptors that fine-tune signaling; as well as on the concentration of these MAMPs. To date, several MAMPs of immunobiotics have been identified, that can be connected to specific host-responses (Lebeer, Vanderleyden, & De Keersmaecker, 2010) and these effector molecules are in many cases associated with the bacterial cell wall (Kleerebezem et al., 2010). Although most beneficial effects of probiotics require direct bacteria-cell contact with live bacteria, some reports demonstrated that soluble factors secreted by lactobacilli are able to modulate the production of cytokines and therefore, to modulate the immune system. In fact, recent investigations have exposed some of the underlying mechanisms in the modulation of the immune system by lactobacilli soluble factors (Mechoud et al., 2012a; Peña & Versalovic, 2003; Seth, Yan, Polk, & Rao, 2008). Therefore, we next evaluated whether the extracellular products present in the CM of lactobacilli exert some immunoregulatory properties. We observed that CM of CRL1014, CRL1101 and CRL1190 significantly increased the production of IL-10 in PBMC in non-inflammatory conditions (Table 1). After the challenge with LPS the CM of CRL1014, CRL1505, CRL75 and CRL1190 did not modified the values of TNF-α when compared to controls, however CM of *L. reuteri* CRL1101 significantly reduced the levels of this cytokine (Table 1). In addition, CM of CRL1505, CRL1101 and CRL1190 increased IL-10 in LPS-challenged PBMC, however the levels of this cytokine were significantly higher in CM CRL1101-treated cells when compared with the two other strains (Table 1).

According to the above results, the strain with the highest capacity to modulate TNF-α and IL-10 production in PBMC in both non-inflammatory and inflammatory conditions was *L. reuteri* CRL1101. Then, this strain was selected for further experiments. To characterize the anti-inflammatory activity of *L. reuteri* CRL1101, PBMC were incubated with the live bacteria or its CM for additional 8 and 24 h. The strain, either as a live bacteria or its CM, was able to induce the release of IL-10 to the culture medium and the levels of this cytokine increased with the time of incubation in non-inflammatory conditions (Fig. 1A). In addition both live *L. reuteri* CRL1101 and its CM increased the levels of TNF-α after 24 h of incubation (Fig. 1A). The CRL1101-CM retained the ability to significantly reduce the TNF-α release by LPS-challenged PBMC at h 8 but not at h 24 (Fig. 1B). Both live *L. reuteri* CRL1101 and its CM were able to improve the IL-10 production in LPS-challenged cells after 8 and 24 h of incubation (Fig. 1B).

It has been discussed about whether probiotics must remain viable and multiply in the gut of the host to be efficient, and which bacterial compounds, such as those from the cell walls or released soluble factors are important in each specific immunological purposes (Corthesy et al., 2007). The precise molecular mechanisms governing the interaction between these beneficial bacteria driven components and the host remain to be discovered, however it is expected that multiple interactions between probiotic molecules and host receptors determined the final immune performance of each

Table 1 – Levels of TNF-α (ng/ml) and IL-10 (pg/ml) released to the culture supernatant by PBMC after 4 h of incubation with live lactic acid bacteria or their metabolic products present in their conditioned media. The results represent three independent experiments. Different alphabet indicates significance ($P < 0.05$).

Stimuli	Strains	Control PBMC		LPS treated PBMC	
		TNF-α	IL-10	TNF-α	IL-10
RPMI	None	0.06 ± 0.03^{a}	2.7 ± 1.6^{a}	1.82 ± 0.76^{bc}	49.3 ± 17.2^{bc}
Live bacteria	CRL1014	1.97 ± 0.48^{c}	12.6 ± 3.5^{abc}	3.51 ± 1.60^{d}	16.2 ± 8.1^{a}
	CRL1505	2.23 ± 0.70^{cd}	19.5 ± 4.9^{abc}	2.92 ± 1.05^{cd}	26.4 ± 17.1^{ab}
	CRL75	0.70 ± 0.31^{b}	18.8 ± 8.6^{abc}	1.45 ± 0.89^{ad}	21.2 ± 15.1^{ab}
	CRL1101	2.44 ± 0.59^{d}	83.8 ± 48.2^{d}	3.45 ± 1.31^{d}	59.4 ± 25.0^{c}
	CRL1190	2.76 ± 0.86^{d}	8.4 ± 8.0^{abc}	3.49 ± 1.17^{d}	13.0 ± 3.8^{a}
Conditioned media	CRL1014	0.54 ± 0.18^{b}	18.0 ± 5.6^{abc}	1.85 ± 0.29^{abc}	37.6 ± 16.7^{bc}
	CRL1505	0.41 ± 0.28^{ab}	3.8 ± 3.4^{ab}	1.16 ± 0.87^{ab}	68.6 ± 21.8^{c}
	CRL75	0.36 ± 0.22^{ab}	7.6 ± 5.9^{abc}	1.64 ± 0.97^{ab}	65.0 ± 13.7^{c}
	CRL1101	0.25 ± 0.10^{ab}	28.3 ± 12.7^{c}	0.63 ± 0.35^{a}	118.8 ± 50.3^{d}
	CRL1190	0.49 ± 0.20^{ab}	27.3 ± 21.3^{ab}	1.38 ± 0.79^{ab}	66.2 ± 28.0^{c}

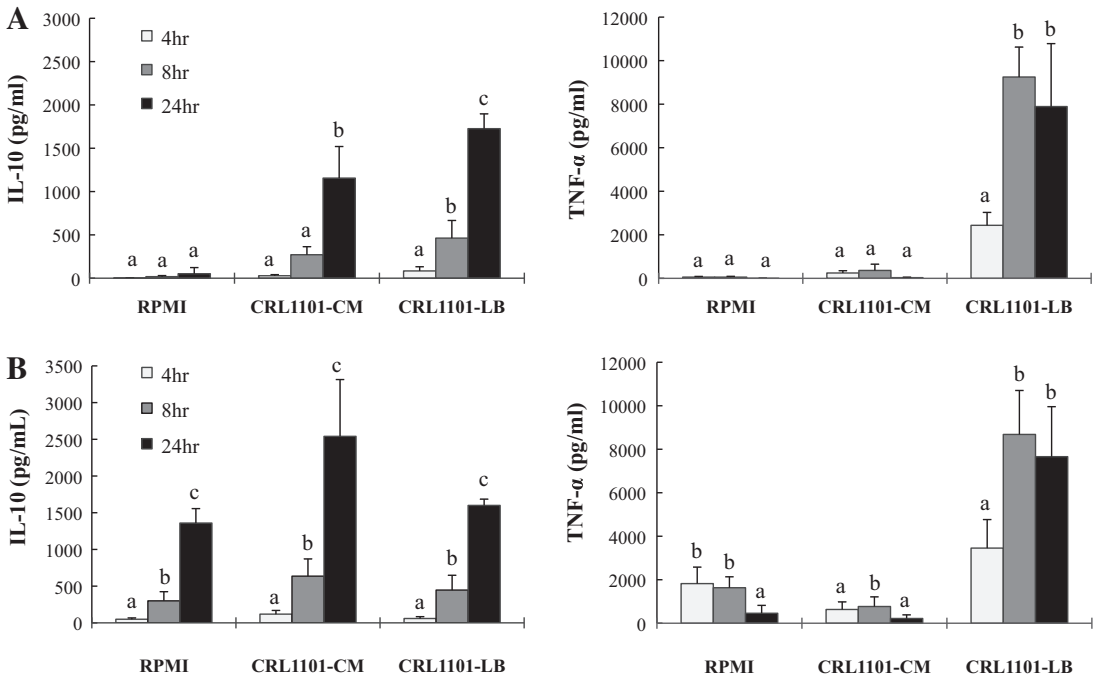

Fig. 1 – Effect of *L. reuteri* CRL1101 on cytokines production by PBMC at different time points. PBMC (4×10^{6}/ml) were incubated in RPMI 1640 medium (control), with viable *L. reuteri* CRL1098 (CRL1101-LB) or its conditioned media (CRL1101-CM) for 4, 8 and 24 h. TNF-α and IL-10 production was measured in culture supernatants by ELISA in non-inflammatory conditions (A) or after LPS (0.5 μg/ml) stimulation (B). Each value represents the mean ± SD obtained from at least three independent experiments. Different alphabet indicates significance ($P < 0.05$).

strain (Corthesy et al., 2007; Lebeer et al., 2010). It seems to be the case for *L. reuteri* CRL1101. According to our results, we can speculate that MAMPs present in both the cell structure of *L. reuteri* CRL1101 as well as their secreted molecules act synergistically to induce a tolerogenic profile in immune cells.

3.2. *L. reuteri* CRL1101 modulate the response of RAW 264.7 macrophages to LPS challenge

The gut of vertebrates is rich in antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs). These APCs reside underneath the epithelial cell layer in an immature state and are prepared to recognize foreign antigens or invading pathogens. Studies on the precise mechanisms of probiotic action indicate that the immunoregulatory mechanisms behind the positive effects of immunobiotics are related to the modulation of immune cells, such as APCs (Braat et al., 2004; Marranzino et al., 2012; Villena et al., 2012b). Moreover, different probiotic strains affect APC maturation in different ways since cytokine and surface marker expression in APCs varies with the probiotic strains used (Christensen, Frøkiaer, & Pestka, 2002; Villena et al., 2012b).

Table 2 – Levels of TNF-α (ng/ml), IL-6 (pg/ml), IL-10 (pg/ml) and nitric oxide (NO, μM) released to the culture supernatant by RAW 264.7 cells after 16 h of incubation with live *Lactobacillus reuteri* CRL1101 (LB) or its conditioned médium (CM). The results represent three independent experiments. Different alphabet indicates significance ($P < 0.05$).

Stimuli	TNF-α	IL-6	IL-10	NO
Control RAW 264.7				
RPMI	0.19 ± 0.08^{a}	2.4 ± 2.8^{a}	22.8 ± 8.9^{a}	0.1 ± 0.1^{a}
CRL1101-LB	10.16 ± 4.70^{c}	107.4 ± 87.1^{c}	161.2 ± 81.5^{c}	2.9 ± 0.3^{b}
CRL1101-CM	3.21 ± 0.70^{b}	14.4 ± 6.7^{b}	55.7 ± 15.1^{b}	0.1 ± 0.1^{a}
LPS treated RAW 264.7				
RPMI	26.42 ± 3.28^{b}	1390 ± 320^{b}	122.7 ± 78.9^{a}	5.0 ± 0.7^{b}
CRL1101-LB	16.96 ± 6.68^{a}	1055 ± 360^{ab}	112.4 ± 55.7^{a}	5.3 ± 1.3^{b}
CRL1101-CM	18.23 ± 6.17^{a}	670 ± 170^{a}	139.1 ± 81.2^{a}	3.5 ± 0.5^{a}

In view of the critical importance of APC polarization in immunoregulation and to further characterize the *in vitro* anti-inflammatory properties of live *L. reuteri* CRL1101 and its CM, the murine macrophage cell line RAW 264.7 was used. In non-inflammatory conditions we observed that *L. reuteri* CRL1101 was able to significantly increase the production of TNF-α, IL-6, IL-10 and NO in RAW 264.7 macrophages while CM of CRL1101 strain increased only the production of TNF-α, IL-6 and IL-10 (Table 2). When we evaluated the effect of the CRL1101 strain in inflammatory conditions we observed that viable *L. reuteri* CRL1101 diminished TNF-α production in LPS-treated macrophages while the its CM was able to decrease the levels of TNF-α, IL-6 and NO released to the culture supernatant after 16 h of incubation (Table 2). These results demonstrated again that both MAMPs present in the cell structure of *L. reuteri* CRL1101 as well as their secreted molecules can modulate macrophages function and induce an anti-inflammatory profile.

The capacity of *L. reuteri* CRL1101 to modulate the response of APC such as macrophages to LPS challenge could have an important impact *in vivo*. It has been showed that probiotics are able to modulate macrophages function in both pro- and anti-inflammatory directions. The capacity of some probiotics strain to stimulate the production of pro-inflammatory cytokines has been considered a positive property when selecting immunostimulatory strains (Marranzino et al., 2012). On the contrary, when searching for anti-inflammatory probiotics, strains with the capacity to upregulate IL-10 and/or reduce TNF-α production are desired (Chiu et al., 2013; Villena et al., 2012b). Several studies showed that LAB can modulate cytokine production by macrophages and suppress TNF-α production by LPS activated human or murine monocytes/macrophages (Chiu et al., 2013; Jones & Versalovic, 2009; Lin, Thibodeaux, Peña, Ferry, & Versalovic, 2008). Moreover, these studies showed that the immunoregulatory effects of probiotic are achieved by contact-dependent and -independent mechanisms. It was reported that *Lactobacillus* GG-conditioned media decreases TNF-α production of Helicobacter-conditioned media-activated peritoneal macrophages (Peña & Versalovic, 2003). It was also shown that the conditioned media of *L. reuteri* strain 6475 has the ability to suppress TNF-α production in activated cells of two different human monocytoid cell lines and primary monocyte-derived macrophages from children and adolescents with Crohn's disease (Lin et al., 2008). Then, as overproduction of pro-inflammatory cytokines, especially TNF-α, is implicated in the pathogenesis of several inflammatory diseases, inhibition of pro-inflammatory cytokine production and alteration of cytokine profiles by *L. reuteri* CRL1101 may highlight an important immunomodulatory role for this strain.

3.3. *Oral administration of L. reuteri CRL1101 protect mice from LPS challenge*

It is known that anti-inflammatory probiotics strains can interact with cells in the gut and induce an anti-inflammatory environment that favors the promotion and development of tolerogenic APCs and $CD4^{+}CD25^{+}Foxp3^{+}$Treg cells, which are important to avoid unproductive inflammation through the production of IL-10 (Shimazu et al., 2012; Villena et al., 2012a, 2012b). Moreover, it was also demonstrated that IL-10 induced by some orally administered probiotic strains is able to reach blood and distant mucosal sites and therefore modulate inflammatory responses in sites distant from the gut (Salva et al., 2010; Villena et al., 2012b). In addition, Ménard et al. (2004) reported that secreted metabolites produced by LAB can cross the intestinal epithelial cell barriers and retain their anti-inflammatory activity. Thus, it is possible to think that even when *L. reuteri* CRL1101 is confined to the intestinal environment after oral administration, it could interact with intestinal cells and induce the release of IL-10 and/or its metabolic product would penetrate the intestinal barrier and reach distant location inducing an anti-inflammatory effect. Therefore, in order to evaluate the immunoregulatory properties of *L. reuteri* CRL1101 *in vivo*, an oral preventive administration of this microorganism was performed followed by endotoxic shock induction, as a model of systemic alteration of the immune response. Different groups of mice were challenged with 2.5, 10, 20 or 30 mg/kg of LPS and the dose of 20 mg/kg was selected for further experiments (data not shown). As was previously reported by Arribas et al. (2009) this dose of LPS does not induce the death of mice in the following 24 h. Then, male BALB/c mice were orally treated with different doses and periods of *L. reuteri* CRL1101 and then received an intraperitoneal injection of LPS from *E. coli*. Preliminary experiments evaluating general health status showed that treatment with 10^{8} cells/mouse/day during 7 days was the optimal dose to protect LPS-challenged mice (data not shown).

Endotoxic shock is complex and heterogeneous syndrome that can be replicated in experimental animal models using

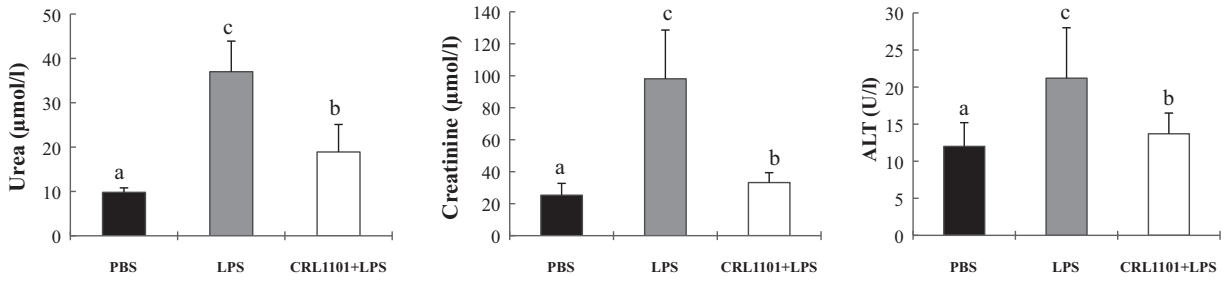

Fig. 2 – Effect of *Lactobacillus reuteri* CRL1101 on lung, kidney and liver damage in LPS-induced septic mice. Male BALB/c mice were fed 2×10^8 UFC/ml CRL1101 bacterial suspension in drinking water for 7 days before the challenge with an intraperitoneal injection of 20 mg/kg LPS. Blood samples were obtained by cardiac puncture 24 h after injection to evaluate the levels of serum urea, creatinine and alanine aminotransferase (ALT) by using clinical assay kits. Results are expressed as means ± SD obtained from at least three independent experiments. Different alphabet indicates significance ($P < 0.05$).

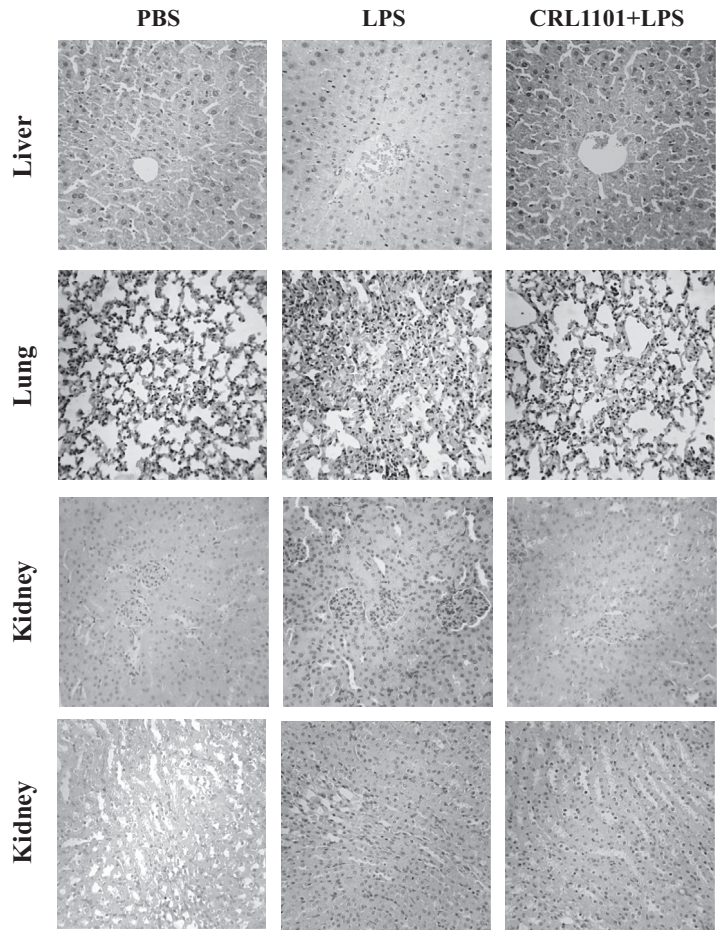

Fig. 3 – Effect of *Lactobacillus reuteri* CRL1101 on lung, kidney and liver tissue damage in LPS-induced septic mice. Male BALB/c mice were fed 2×10^8 UFC/ml CRL1101 bacterial suspension in drinking water for 7 days before the challenge with an intraperitoneal injection of 20 mg/kg LPS. Lung, kidney and liver samples were obtained 24 h after the injection of LPS. Histological analysis stained with HE.

administration of LPS. Lethal doses of LPS elicited a systemic inflammatory process of the immune system to release pro-inflammatory mediators, such as IL-1β, IL-6, IL-8, TNF-α and platelet-activating factor, as well as nitric oxide (NO) and reactive oxygen species (ROS) which are associate with oxidative stress and damage. These overwhelming pro-inflammatory mediators result in hepatic and renal failure, myocardial dysfunction, acute respiratory distress syndrome and disseminated intravascular coagulation which lead to death (Lappin & Ferguson, 2009). Inhibition of the pro-inflammatory cytokine production especially TNF-α and IL-1β, or the improvement of IL-10 have been developed as important tar-

gets for management of severe sepsis and its derivative of multi-organ failure (LaRosa & Opal, 2008; Orlicek, 2001). Then, to study the anti-inflammatory effect of *L. reuteri* CRL1101 we evaluated markers of organ failure and cytokines concentrations in blood.

Although systemic administration of LPS causes a multi organ failure, lungs, kidneys and liver are largely affected (Hotchkiss & Karl, 2003). Therefore, we focused our studies in those organs. Plasma levels of urea and creatinine were used to evaluate kidney function. Both, urea and creatinine significantly increased in plasma after the challenge with LPS (Fig. 2). These parameters were significantly lower in mice preventively treated with *L. reuteri* CRL1101. Serum alanine-aminotransferase (ALT) and aspartate aminotransferase (AST) activities were also evaluated as liver function markers. Both, ALT and AST significantly increased in serum after the challenge with LPS (Fig. 2). ALT activity was lower in septic mice pre-treated with the CRL1101 strain compared with the control group (Fig. 2), while AST levels were not significantly different between the groups (data not shown). In addition, histological studies were performed in lung, liver and kidneys in order to confirm tissue damage induced by LPS challenge. As shown in Fig. 3, systemic administration of LPS clearly altered the normal histological structure of lung, liver and kidneys with an evident infiltration of pro-inflammatory cells. These alterations were significantly lower in mice preventively treated with *L. reuteri* CRL1101 (Fig. 3).

Since pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β are crucial in the development of sepsis, causing intravascular disseminated coagulation, vascular failure and shock (Lappin & Ferguson, 2009), serum values of these cytokines were investigated. As expected, the challenge with LPS significantly increased blood levels of TNF-α, IL-6 and IL-1β (Fig. 4). However, treatment with the CRL1101 strain significantly reduced blood concentrations of TNF-α and IL-6 but not IL-ß (Fig. 4).

Studies have shown that an LPS-induced up-regulation of TNF-α release is closely followed by enhanced secretion of IL-10 as a counter-regulatory cytokine. Studies have demonstrated that IL-10 can protect mice from lethal endotoxemia when administered concomitantly or following LPS challenge (Annane, Bellissant, & Cavaillon, 2005). The anti-inflammatory mechanisms, including the release of IL-10, are also activated in sepsis as intent to control and reduce the inflammatory damage, but there is a clear predominance of the inflammatory response (Annane et al., 2005). For these reasons, more efficient strategies to potentiate these subliminal anti-inflammatory pathways, which could modulate the inflammation, are needed. When we analysed IL-10 concentration in LPS-challenge mice we observed increases of this cytokine in serum. Mice previously treated with *L. reuteri* CRL1101 showed significantly higher levels of IL-10 than control LPS-challenged mice (Fig. 4). Our results are in accordance with findings for other probiotic strains. It was reported that the administration of the probiotic *L. salivarius* ssp. *salivarius* CECT5713 clearly improved the altered immune response and decreased the production of inflammatory mediators associated to endotoxic shock induced by LPS. The probiotic was able to downregulate the increased levels of TNF-α both in plasma and lungs (Arribas et al., 2012). When the production of IL-10 was evaluated in mice splenocytes stimulated with Con A, a decrease was observed in control mice when compared to the healthy group, thus reflecting the failure of this anti-inflammatory cytokine to control systemic inflammatory situation following the upregulated release of pro-inflammatory cytokines. Similarly to our work, the

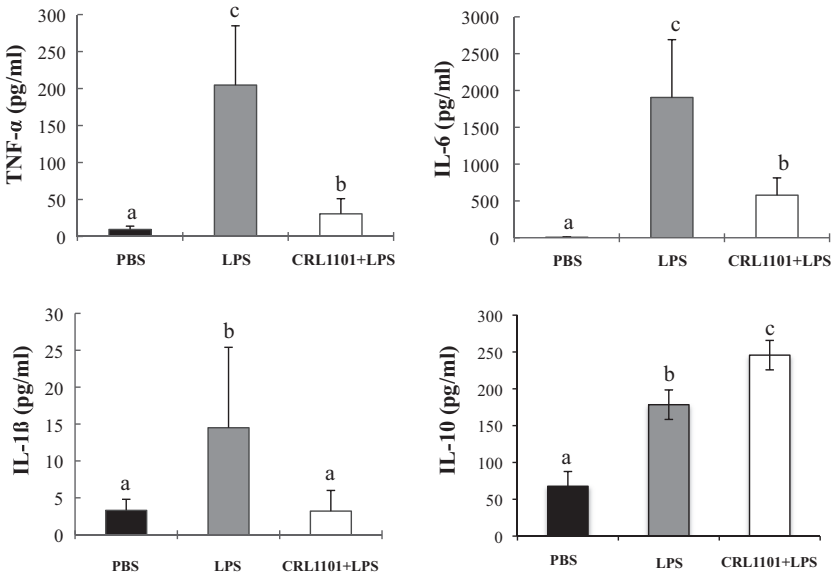

Fig. 4 – Effect of *Lactobacillus reuteri* CRL1101 on blood cytokines levels in LPS-induced septic mice. Male BALB/c mice were fed 2×10^8 UFC/ml CRL1101 bacterial suspension in drinking water for 7 days before the challenge with an intraperitoneal injection of 20 mg/kg LPS. Blood samples were obtained by cardiac puncture 24 h after injection to evaluate the levels of serum TNF-α, IL-6, IL-1β and IL-10 by using ELISA assays. Results are expressed as means ± SD obtained from at least three independent experiments. Different alphabet indicates significance ($P < 0.05$).

pretreatment of septic mice with the CECT5713 strain resulted in a significant increased production of IL-10, thus contributing to the downregulation of pro-inflammatory cytokines, including TNF-α, observed after probiotic treatment to septic mice (Arribas et al., 2012). Then, the results of the present work show that *L. reuteri* CRL1101 is able to beneficially modulate the balance of pro- and anti-inflammatory cytokines during sepsis similarly to other probiotic strains.

3.4. *Oral administration of L. reuteri CRL1101 protect mice from tissue oxidative damage*

In recent years, some of the postulated molecular mechanisms of sepsis progression are related to the imbalance between ROS production and scavenging by the cellular antioxidant system (Bitzer-Quintero et al., 2010; Rinaldi, Landucci, & De Gaudio, 2009). Lots of studies have shown that excessive production of ROS and other free radicals in sepsis, such as H_2O_2, superoxides and NO, which are associated with inflammation, lead to a condition of oxidative stress and contribute to high mortality rates (Lowes, Thottakam, Webster, Murphy, & Galley, 2008). However, direct detection of ROS and other free radicals is quite difficult since they are all short-lived and highly reactive in a nonspecific manner (Kohen & Nyska, 2002). Thus, oxidative damage is generally analyzed by measurement of secondary products including derivatives of amino acids, nucleic acids, and lipid peroxidation. In this work, in order to evaluate the potential effect of *L. reuteri* CRL1101 in endotoxic shock associated-tissue oxidative damage, indicators of oxidative stress were measured in tissue homogenates.

Lung MPO activity was used as marker of neutrophils infiltration (Racedo et al., 2006). In our mice model the MPO activity in lungs significantly increased after the challenge with LPS (Fig. 5). We also observed that lung MPO was significantly lower in mice treated with *L. reuteri* CRL1101 when compared with control mice (Fig. 5). As liver is a major site of antioxidant activities in the body, we evaluated TBARS as products of lipid peroxidation in liver homogenates. Again, challenge with LPS increased liver TBARS and treatment with *L. reuteri* CRL1101 significantly reduced them (Fig. 5). Another parameter of oxidative damage, the non-protein sulphydryl group (GSH) concentration, was also evaluated in liver homogenates. LPS treatment caused a reduction in the GSH levels, while administration of the CRL1101 strain increased the levels of GSH that reached the normal values (Fig. 5).

Many studies suggest that oxidative stress and inflammation can interact in many conditions. Reactive nitrogen and oxygen species resulting from uncontrolled activation of macrophages and neutrophils are important players in the pathogenesis of endotoxic shock and sepsis causing lipid peroxidation of membranes, antioxidant system depletion and organ dysfunction (Rinaldi et al., 2009). A growing number of studies have demonstrated that various ROS scavengers have been shown to ameliorate multiple organ dysfunctions and thus improve survival rate in various animal models of sepsis or endotoxemia (Liu, Kao, & Chen, 2008). It was shown that when a ROS scavenger such as vitamin C is used in septic mice, reduced organ injury, improved organ dysfunction, increased antioxidant enzymatic activities and improved

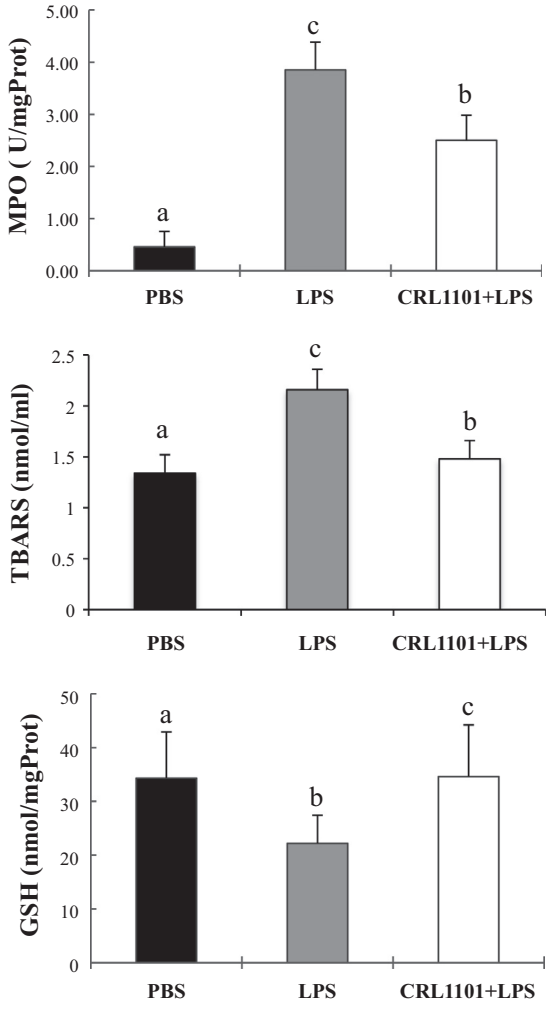

Fig. 5 – Effect of *Lactobacillus reuteri* CRL1101 on LPS-induced oxidative damage. Male BALB/c mice were fed 2×10^8 UFC/ml CRL1101 bacterial suspension in drinking water for 7 days before the challenge with an intraperitoneal injection of 20 mg/kg LPS. Lungs and livers were collected 24 h after injection to evaluate lung myeloperoxidase (MPO) activity, liver TBARS and non-protein sulphydryl group (GSH) concentrations in organs homogenates. Results are expressed as means ± SD obtained from at least three independent experiments. Different alphabet indicates

survival rates are found (Hou et al., 2009). Moreover, it was demonstrated that ROS scavengers in addition to their capacity to increase the activities of antioxidant enzymes, they are able to improve the levels of IL-10 and decrease TNF-α and other pro-inflammatory cytokines (Hou et al., 2010).

Archibald and Fridovich (1981) have observed that LAB can deal with oxygen radicals by either SOD or higher internal Mn^{2+} concentration. In addition, it is also believed that during transit through the gastrointestinal tract, lactobacilli may release their intracellular antioxidative constituents that may reduce tissue injury and thereby protect the host (Lin, Chyi, Wu, Hwang, & Ho, 1998). Our results show that *L. reuteri* CRL1101 preventive administration could reduce the damage associated to the redox imbalance. These results are in line

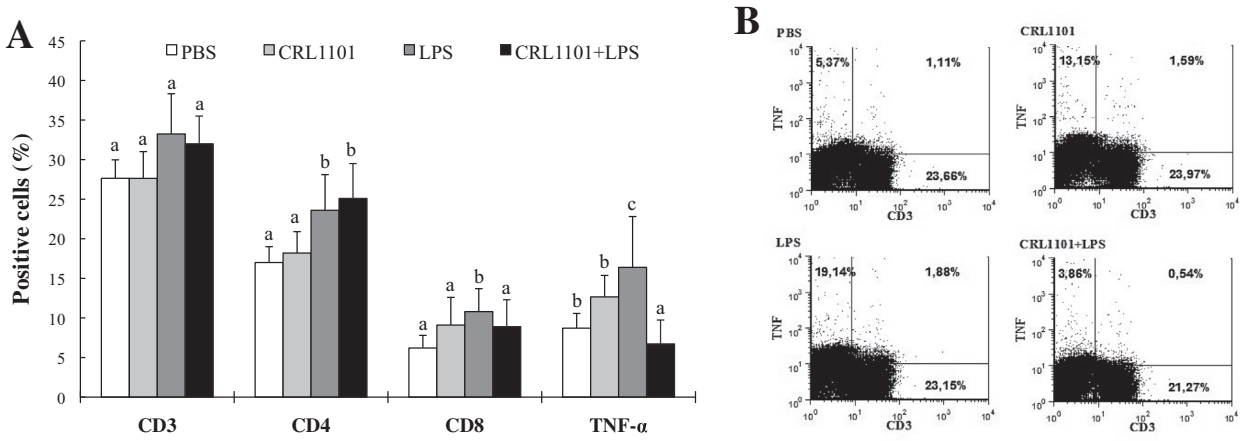

Fig. 6 – Effect of *Lactobacillus reuteri* CRL1101 on the splenocytes immunophenotype in LPS-induced septic mice. Male BALB/c mice were fed 2×10^8 UFC/ml CRL1101 bacterial suspension in drinking water for 7 days before the challenge with an intraperitoneal injection of 20 mg/kg LPS. Spleens were aseptically removed 24 h after the challenge with LPS. Single cell suspensions of splenocytes were obtained and prepared for flow cytometry staining. CD3, CD4, CD8 and TNF-α positive cell populations were detected by flow cytometry (A). Histograms of TNF-α versus CD3 for each experimental group is shown in panel B. Results are expressed as means ± SD obtained from at least three independent experiments. Different alphabet indicates significance ($P < 0.05$).

with previous reports about the potential antioxidant properties of probiotics strains selected by their *in vitro* antioxidant activity which showed the capacity to prevent oxidative damage in a doxorubicin-induced oxidative stress model in rats (Amaretti et al., 2012). To find out the components of the CRL1101 strain that are responsible for the antioxidant activity is an interesting point for future investigations.

3.5. *Effect of L. reuteri CRL1101 on splenocytes immunophenotyping*

Since spleen is a lymphoid organ that is in close contact with the systemic circulation, immumophenotypes of spleen cells were analyzed to explore the effect of *L. reuteri* CRL1101 on systemic immune cells. Then, we evaluated the effect of *L. reuteri* CRL1101 on the percentage of CD3, CD4, CD8, INF-γ and TNF-α positive cells in spleen in non-inflammatory and inflammatory conditions. Treatment of mice with *L. reuteri* CRL1101 did not modified the number of CD3, CD4 or INF-γ positive cells. However, the CRL1101 strain produced a slight increase of CD8 and TNF-α positive cells in healthy mice (Fig. 6A). Mice undergoing sepsis showed a significant increase on CD4, CD8 and TNF-α positive cells in spleen when compared with healthy mice. Treatment with *L. reuteri* CRL1101 decreased CD8 and TNF-α positive cells compared with control group (Fig. 6A). We also demonstrated that changes in TNF-α positive cells are related to variations in cells not expressing the CD3 marker (Fig. 6B). Therefore, changes in spleen TNF-α were related to variations in the myeloid cell population.

Host reaction to systemic sepsis and endotoxic shock is associated with a wide variety of modifications to the immune system. An overall decrease in T cell proliferation is observed and profound depression of T cell-dependent immunity has been demonstrated following induction of polymicrobial sepsis in rodent models (Pape, Remmers, Grotz, & Tscherne, 1999). Moreover, during sepsis, initially cells of the Th1 phenotype predominate. Following this, increasing concentrations of Th2 cells are observed, accompanied by a rising plasma IL-4 and IL-10 concentration and a falling concentration of IL-2 and IFN-γ (known as the Th1–Th2 shift). This changes produce marked overall suppression of immune responsiveness in Th1 $CD4^+$ lymphocytes demonstrated by reduced cytokine synthesis and an increased rate of apoptosis (Hildebrand et al., 2004). Most of these changes have been attributed to TNF-α increase (Hildebrand et al., 2004). In addition, it was observed a marked increase in the proportion of $CD8^+$ cells following induction of endotoxic shock and these $CD8^+$ cells have been shown to augment the pro-inflammatory response and promote mortality (Sherwood, Enoh, Murphey, & Lin, 2004; Sherwood et al., 2003). Then, we were able to find all the expected changes for endotoxic shock in our mice model. And more importantly, we were able to demonstrate that the preventive administration of *L. reuteri* CRL1101 significantly reduced CD8 and TNF-α positive cells during sepsis and therefore we showed an immune mechanism that would be involved in the beneficial effect of this strain.

4. Conclusion

The present study supports the proposed idea that, although probiotic microorganisms may exert their beneficial effects due to their well-known ability to modulate intestinal immunity, these effects are not restricted to the gastrointestinal tract. Moreover, this work supports the hypothesis that selected probiotic strains or their specific extracellular products may be helpful to control systemic excessive inflammatory responses. We showed that *L. reuteri* CRL1101 can reduce inflammatory and oxidative damage during endotoxic shock, and that this beneficial effect is probably related to the capacity of the MAMPs present in both the cell structure as well as their secreted molecules to act synergistically to induce a tolerogenic profile in intestinal and systemic immune cells. The

previous and the present studies of our laboratory support the findings that probiotic strains may secrete immunomodulatory factors, or immunomodulins, that suppress pro-inflammatory cytokines production and exert a systemic anti-inflammatory effect. Although the identities of these factors have not been deciphered to date, our group is working actively to determine the nature of those immunomodulins.

This work also demonstrated that the *in vitro* screening of LAB strains with specific properties is still a powerful approach to discriminate the potential probiotic bacteria for specific functions that could be later tested and studied in animal models. From the systematic studies reported here, *L. reuteri* CRL1101 a strain with technological potential usefulness in dairy products, is proposed as novel probiotic candidate for the prevention and control of systemic inflammatory disorders.

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