Lactobacillus reuteri CRL1098 soluble factors modulate tumor necrosis factor alpha production in peripheral blood mononuclear cells: Involvement of lipid rafts

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ABSTRACT

The aim of the present study was to evaluate the capacity of Lactobacillus reuteri CRL1098 soluble factors (Lr-S) to modulate TNF-α production in peripheral blood mononuclear cells (PBMC) and to study lipid rafts participation in this response. PBMC treated with Lr-S showed a reduced production of TNF-α. In addition, Lr-S treatment activated ERK and p38 MAPK pathways in PBMC. Lipid rafts participation in the reduced production of TNF-α by PBMC induced by Lr-S was verified by lipid rafts disruption with methyl-β-cyclodextrin and the reduction of the Src-tyrosine kinase Lck localization in rafts. Moreover, PBMC pre-treatment with Lck inhibitors blocked the effect of Lr-S on TNF-α production suggesting that activation and mobilization of Lck from lipid rafts would be involved in the modulatory effect of L. reuteri CRL1098. A secreted peptide of 5785 Da would be responsible of the modulatory effect of CRL1098 strain. This study demonstrated for the first time the lipid rafts participation in a response induced by a beneficial bacterium. Also, these results open new possibilities for investigating the molecular mechanisms involved in the interaction of probiotic bacterial extracellular compounds with immune cells.

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1. Introduction

Lactic acid bacteria (LAB) are normal habitants of the human gastrointestinal tract and they are main components of the microbiota of the small intestine. They are considered beneficial to the host and have been widely used as probiotic, defined as "live microorganisms which when administered in adequate amounts confer health benefits to the host" [1]. Some lactobacilli strains were reported to exert in vitro stimulatory properties on cells of the immune system, including monocytes and macrophages, and to induce the production of both pro-inflammatory and regulatory cytokines, such as IL-6, TNF-α and IL-10 [2–5]. Moreover, a large number of experimental and clinical studies published in recent years have shown the beneficial role of probiotic LAB in the immune status of the host. However, because the different receptors of the innate immune system can recognize only specific bacterial molecular patterns, knowledge of the role played by individual probiotic molecular patterns is essential to move from the current confused era of live probiotic bacteria to the era of the pharmacobiotic strategies [6]. The communication between probiotic LAB and host cells is multifactorial and involves an integrative repertoire of receptors on the host side that recognize multiple effector molecules on bacteria, of which most have been found to be cell wall- or cell surface-associated compounds and proteins [7]. Although most beneficial effects of probiotics require direct bacteria-cell contact with live bacteria [8], some reports demonstrated that soluble factors secreted by lactobacilli are able to modulate the production of cytokines and therefore, to modulate the immune system [2,4,5,9,10]. However, the underlying mechanisms regulating the cytokines modulation by LAB soluble factors are incompletely understood.

Many pathogenic bacteria have been found to interact with specialized domains of host cell plasma membrane, termed as lipid rafts [11]. These small and highly dynamic structures of eukaryotic cells are considered signaling platforms because they concentrate molecules involved in key signaling pathways. Lipid rafts modulate several responses in immune cells, for example, TNF-α secretion in lipopolysaccharide (LPS)-activated macrophages depends on proteins concentrated in these microdomains [12]. Biochemically, lipid rafts are specifically enriched in cholesterol and glycosphingolipids. Due to their organization, tight packaging between the sterol and saturated acyl chains, lipid rafts restricts the access of proteins in such a way

that only proteins attached to the membrane by a lipid anchor, such as glycophosphatidylinositol (GPI)-anchored proteins or acylated cytosolic proteins segregate within the these microdomains [13]. A large variety of proteins have been detected in lipid rafts isolated from different cell types including Src-like tyrosine kinases (Lck, Lyn, Fyn), Flotillin and linker for activated T cells (LAT) [14,15].

Interactions of non-pathogenic bacteria such as LAB with these microdomains have just begun to be studied [16]. In this sense, we demonstrated that some lactobacilli strains modulate TNF-α production by human peripheral blood mononuclear cells (PBMC), and that cell response depended on lipid rafts integrity [16]. The capacity of *L. acidophilus* CRL1014, *L. rhamnosus* CRL1036 and *L. reuteri* CRL1098 to modulate TNF-α production by PBMC was investigated and we found that cytokine secretion was modified by the three strains in different ways. *L. acidophilus* and *L. rhamnosus* induced secretion of TNF-α. In contrast, in co-cultures of PBMC with *L. reuteri* CRL1098, TNF-α secretion was reduced. Moreover, the inhibitory effect of *L. reuteri* on TNF-α production was increased in disrupted rafts PBMC as compared to the control cells [16]. Therefore, we became interested in *L. reuteri* CRL1098 because its capability to downregulate the production of a pro-inflammatory cytokine with rafts involvement.

The aim of this study was to determine whether *L. reuteri* CRL1098 secretes soluble factors that modulate the pro-inflammatory cytokine (TNF-α) production in human PBMC and to define lipid rafts participation on this effect.

2. Materials and methods

2.1. Bacteria cultures and supernatant obtaining

Lactobacillus reuteri CRL1098 (provided by the Culture Collection of Centro de Referencia para Lactobacilos, CERELA. Tucumán, Argentina) was grown in MRS (de Man, Rogosa and Sharpe medium, Britania, Buenos Aires, Argentina) up to mid logarithmic growth phase at 37 °C (OD at 560 nm of approximately 0.50). The culture was centrifuged (8000 $\times g$ for 10 min), washed with phosphate-buffered saline (PBS) and RPMI 1640 medium with phenol red (GIBCO cat. No. 22400, Grand Island, NY, USA), resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) and then propagated 4 h at 37 °C, 5% CO_2 in non-agitating conditions (4×10^7 CFU/ml). The culture was centrifuged and cell-free supernatant was aseptically filtered using 0.22 μm pore size low protein binding cellulose acetate filters (Millipore, Bedford, MA, USA). The cell-free supernatant (pH 7.2) was used as recovered from the filtrates (non-diluted).

2.2. Isolation of PBMC and treatment with L. reuteri CRL1098 supernatant

Human PBMC were isolated from heparinized whole blood from healthy volunteers (range 24–42 years old). Prior inclusion, all healthy volunteers gave their informed consent to participate in the study. PBMC were isolated by density gradient centrifugation with Ficoll-Hypaque (Histopaque-1077 Hybri-Max, Sigma, St. Louis, MO, USA). PBMC were washed with PBS and RPMI 1640 medium and cell viability was determined by Trypan Blue (Sigma, St. Louis, MO, USA) exclusion assay. PBMC (2×10^6 cells) were seeded into 24-well plates (TPP, Switzerland) and incubated with *L. reuteri* supernatant (Lr-S) for 4 h. The culture supernatant was collected for determination of TNF-α and IL-10 levels by enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA, USA). Lck inhibitor PP2 and its inactive analog PP3 (Calbiochem, MA, USA), were incubated at a concentration of 10 μM with the PBMC at 37 °C and 5% CO_2 for 30 min prior to the incubation with *L. reuteri* supernatant.

2.3. Disruption of lipid rafts

PBMC were treated with 10 mM methyl-β-cyclodextrin (MβCD, Sigma, St. Louis, MO, USA) in RPMI 1640 medium without fetal bovine serum as described by Soria et al. [16]. Disruption of lipid rafts was assessed by cholesterol extraction and quantitation [17]. In addition, Filipin (Sigma, St. Louis, MA, USA) staining protocols were used. Disrupted-rafts PBMC were adjusted to 2×10^6 cells/ml in RPMI 1640 medium, treated with Lr-S for 4 h, and the culture supernatant was collected for TNF-α and IL-10 ELISA assay. Results were compared with those obtained for PBMC with intact lipid rafts (control PBMC).

2.4. Lipid rafts isolation

Lipid rafts fractions were isolated from entire PBMC (4×10^7 cells) treated with Lr-S. Lipid rafts were collected by centrifugation based on the procedure previously described by Brown and Rose [18], with slight modifications to our experimental system. Briefly, PBMC were resuspended in 800 μl ice-cold (0–4 °C) lysis buffer (1% (v/v) Triton X-100, 10 mM Tris–HCl pH 7.4, 70 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 2 μg/ml Leupeptin, 1 μg/ml Aprotinin, 1 μg/ml Pepstatin, and 0.1 mM PMSF). After 30 min, an equal volume of 80% (w/v) sucrose in lysis buffer was added. Samples (1.6 ml) were placed in ultracentrifuge tubes, and 30% (w/v) sucrose (6.6 ml) and 5% (w/v) sucrose (2.6 ml) were layered on top. Gradients were then centrifuged at 120,000 $\times g$ for 20 h at 4 °C in a Beckman Coulter Optima L-90 ultracentrifuge. Fractions were subsequently harvested from the top to the bottom of the tube. The raft fraction was centrifuged at 120,000 $\times g$ for 1 h at 4 °C in lysis buffer without Triton X-100 and the pellet containing lipid rafts was resuspended in TBM buffer for further determination of protein content, using the DC protein assay kit (Bio-Rad, Hercules, USA).

2.5. Preparation of whole cells extracts

Whole PBMC extracts were prepared by disrupting the harvested cells in a lysis buffer containing 20 mM HEPES pH 7.5, 2.5 mM $MgCl_2$, 10 mM EDTA, 4% (v/v) Nonidet P-40, 0.5 mM PMSF, 1 mM Leupeptin, 1 mM Aprotinin and 1 mM Pepstatin. Total protein concentrations were quantified using DC protein assay kit (Bio-Rad, Hercules, USA).

2.6. Preparation of cytosolic extracts

Cytosolic extracts were prepared by disrupting the harvested PBMC in a lysis buffer containing 10 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.25% (v/v) Nonidet P-40, 0.5 mM PMSF, 0.5 mM Leupeptin, 1 mM Aprotinin and 1 mM Pepstatin. Total protein concentrations were quantified using DC protein assay kit (Bio-Rad, Hercules, USA).

2.7. Western Blot analyses

Proteins (30 μg) from samples were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and probed with various primary antibodies. Membranes were blocked in 5% (w/v) non-fat milk prior to incubation with the primary antibodies anti-Lck, anti-Flotillin-1, anti-ERK, anti-p-ERK, anti-p-p38, anti-Tubulin and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a peroxidase conjugated secondary antibody (anti-rabbit IgG or anti-mouse IgG). Blots were detected by chemiluminescence (ECL, Amersham Biosciences) using standard X-ray films (Kodak X-Omat AR).

2.8. Characterization of L. reuteri CRL1098 supernatant peptide

L. reuteri supernatant was analyzed with a C8 column (X-Terra MS, 3.9×100 nm $\times 3.5$ μm. Waters, Millford, MA, USA) in RP-HPLC Alliance2695 (Waters, Millford, MA, USA), using an acetonitrile gradient from 0.5 to 5% (v/v) for 5 min, 5 to 60% (v/v) for 60 min and 60 to 100% (v/v) for 10 min. The mobile phase was pumped at a flow rate of 1 ml/min. The analytes were detected with a multiple wavelength detector set at 214 and 280 nm.

Mass spectrometric data were obtained using a MALDI-TOF-TOF spectrometer, Ultraflex II (Bruker), in the mass spectrometry facility CEQUIBIEM, Argentina.

2.9. Statistical analyses

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 ANOVA general linear model followed by Tukey's post-hoc test. Statistically significant differences were defined at a p value < 0.05.

3. Results

3.1. L. reuteri CRL1098 soluble factors decrease TNF-α production

To evaluate the cytokines modulation exerted by L. reuteri CRL1098 soluble factors, PBMC were treated with Lr-S. Supernatant was obtained after 4 h incubation of L. reuteri CRL1098 in RPMI 1640 medium, previously determined as the optimal incubation time at which L. reuteri CRL1098 modulates cytokine production by PBMC [16]. We observed that the treatment of PBMC with Lr-S decreased TNF-α production by 34%. Additionally, we investigated whether Lr-S was able to modulate the production of a regulatory cytokine such as IL-10 in PBMC. No significant modification of IL-10 production was observed in this time point (Fig. 1). These results were consistent with our previous report in which only live L. reuteri was able to decrease TNF-α production, while heat-killed bacteria exerted no effect [16]. Cellular cytotoxicity did not appear to be responsible for Lr-S-dependent suppression of TNF-α production because of cell viability of Lr-S-treated cells was not different from untreated control cells, as determined by Trypan Blue dye exclusion assay (96% viability or higher).

In order to analyze the signaling pathways involved in the effect of L. reuteri, PBMC were incubated with Lr-S and the activation of ERK ½ and p38 MAPK pathways was determined in the cellular extracts by Western Blot assays. The results showed that after 4 h incubation, Lr-S induced activation of both ERK ½ and p38 MAPK in PBMC (Fig. 2A, B). To further determine whether ERK ½ and p38 activation

was related to an increased synthesis of the enzymes, PBMC treated with Lr-S for 4 h were harvested by centrifugation and total ERK ½ and p38 protein levels were measured by ELISA. Results demonstrated that there were no significant changes in the levels of expression of both proteins after 4 h incubation in response to Lr-S (Fig. 2C), indicating that the increased levels of p-ERK and p-p38 induced by Lr-S were related to an activation of these signaling pathways and not to an incremented synthesis of the proteins.

3.2. Lipid rafts are involved in Lr-S effects on PBMC

In order to assess whether lipid rafts were involved in the modulation of TNF-α production induced by Lr-S, PBMC were pretreated with methyl-β-clyclodextrin, which specifically depletes cholesterol and disrupts lipid raft organization. Disruption of lipid rafts in PBMC was confirmed by using cholesterol quantitation and Filipin staining protocols. We observed a marked decrease in cholesterol levels and in fluorescence associated to cholesterol in disrupted-rafts PBMC (data not shown). Subsequently, disrupted-rafts PBMC were incubated with Lr-S. A higher diminution of TNF-α was observed in PBMC treated with Lr-S compared to intact-rafts cells (54 and 32% reduction, respectively, Fig. 3), suggesting the intervention of lipid rafts in this response. As observed in intact-rafts PBMC, Lr-S did not induce changes in IL-10 production by disrupted-rafts PBMC (Fig. 3).

3.3. Lr-S decreases the raft localization of tyrosine kinase Lck

To further investigate the intervention of lipids rafts in the effects induced by Lr-S, PBMC were treated with Lr-S for 4 h and lipid rafts were isolated by sucrose density gradient. Flotillin-1, considered a lipid rafts marker [19] and Lck, constitutively present in the lipid rafts of T cells and involved in signaling pathways [13], were analyzed by Western Blot in total membrane and lipid rafts fractions. Flotillin-1 was not significantly modified by Lr-S neither in total nor in the lipid rafts fractions (Fig. 4A, B). Similarly to Flotillin-1, Lck was concentrated in lipid rafts fractions. However, Lr-S decreased Lck levels in lipid rafts (Fig. 4A, B). Additionally, the effect of Lr-S on Lck levels was evaluated in total cellular lysates and in the cytosolic fractions obtained from PBMC and PBMC incubated with Lr-S. The results showed that Lck localization was not modified by Lr-S neither in the total nor in the cytosolic fractions of the cellular lysates (Fig. 4C, D).

We then used a strategy of Lck pharmacological blockers in order to find out whether the TNF-α reduction induced by Lr-S is related to Lck kinase. PBMC were treated with Lck inhibitor PP2 and its negative analog PP3, prior to the incubation of cells with Lr-S. In PBMC treated with PP2 the diminished TNF-α production induced by Lr-S was abolished, whereas no changes was observed in PBMC treated with inactive analog PP3 (Fig. 5). These results demonstrated that Lck
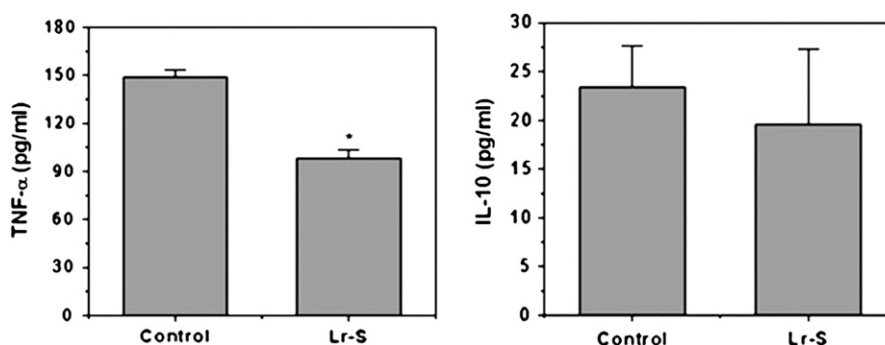

Fig. 1. Effect of L. reuteri CRL1098 on cytokines production in PBMC. PBMC were incubated in RPMI 1640 alone (control) or with L. reuteri CRL1098 supernatant (Lr-S) for 4 h at 37 °C and 5% CO$_2$. TNF-α and IL-10 production was measured in culture supernatants by ELISA. Each value represents the mean ± SD obtained from at least three different experiments. *Significantly different compared to the respective control group ($p < 0.05$, one way ANOVA test).
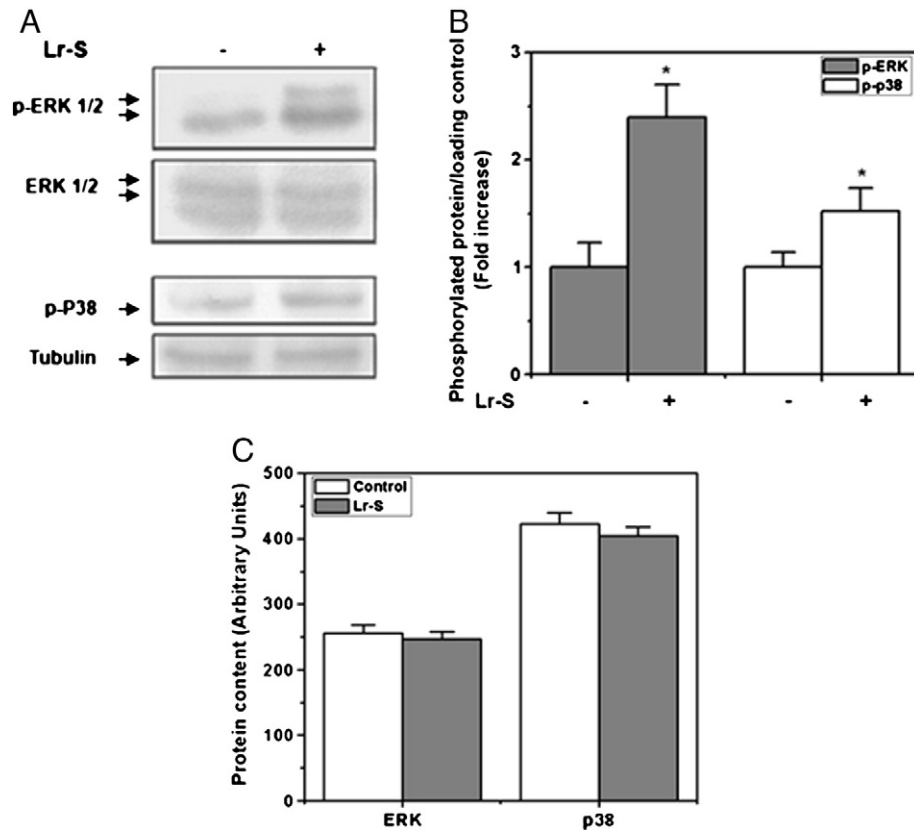
Fig. 2. Effect of *L. reuteri* CRL1098 on MAPK activation in PBMC. (A). ERK ½ and p38 phosphorylation was analyzed by Western Blot. Total protein content was analyzed by using anti-Tubulin and anti-ERK antibodies. One representative Western Blot of three independent experiments is shown. (B) Quantitation of Western Blot of three independent experiments. (C) Total levels of ERK and p38 were measured by ELISA. *Significantly different compared to control PBMC ($p<0.05$, one way ANOVA test).

participates in the reduction of TNF-α production induced by Lr-S in PBMC.

3.4. A peptide from L. reuteri CRL1098 supernatant reduces the TNF-α production by PBMC

To identify the compound released by *L. reuteri* CRL 1098 to the supernatant, involved in the regulation of TNF-α production by PBMC, the bacterial supernatant was subjected to different treatments: heating, freezing/thawing and digestion with Pronase E. Supernatants subjected to these treatments were incubated with control and disrupted-rafts PBMC to evaluate their capacity to modulate TNF-α production. The decreased TNF-α production by control and disrupted-rafts PBMC observed with untreated *L. reuteri* supernatant (Lr-S) was lost after the supernatant treatments and digestion (Fig. 6A). Subsequently, Lr-St was filtered/concentrated through Amicon membranes. The fractions containing compounds between 3 and 10 kDa diminished TNF-α production similarly to non-filtered supernatant. Fraction under 10 kDa induced a reduction of TNF-α by 33 and 44% in control and disrupted-rafts PBMC respectively while the percentages of reduction for the fraction higher 3 kDa was 62 and 68%, respectively (Fig. 6B). No effects were observed in filtrates containing products higher
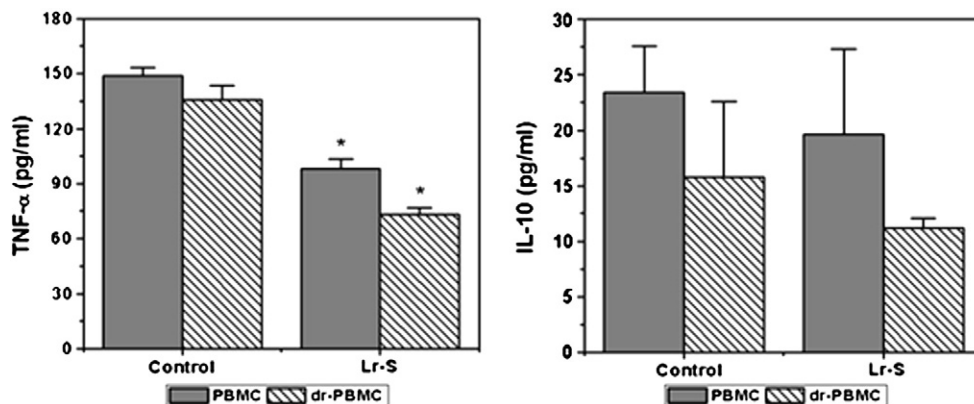
Fig. 3. Effect of *L. reuteri* CRL1098 on cytokines production in disrupted-rafts PBMC (dr-PBMC). PBMC were incubated in RPMI 1640 alone (control) or with *L. reuteri* CRL1098 supernatant (Lr-S) for 4 h at 37 °C and 5% CO_2. TNF-α and IL-10 production was measured in culture supernatants by ELISA. Each value represents the mean ± SD obtained from at least three different experiments. *Significantly different compared to the respective control group ($p<0.05$, one way ANOVA test).
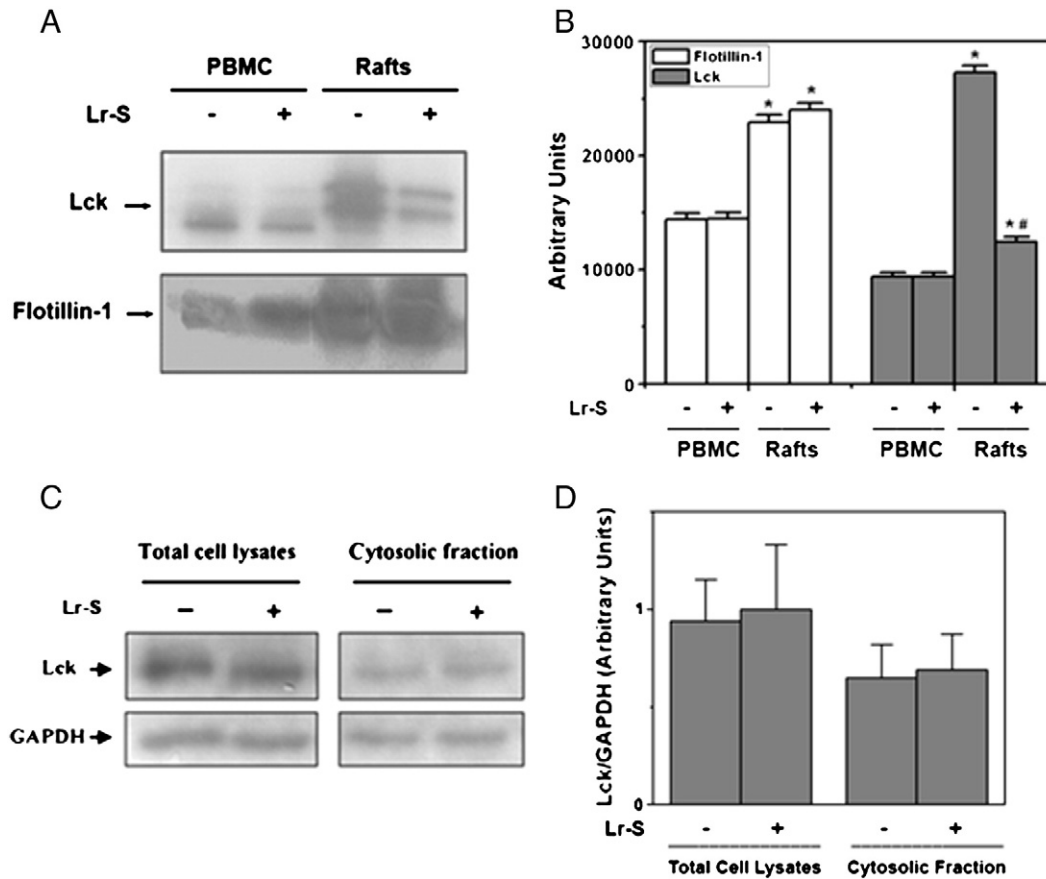
Fig. 4. Protein characterization of lipid rafts isolated from PBMC. (A) Flotillin-1 and Lck content was analyzed by Western Blot in PBMC and their respective isolated lipid rafts after incubation with or without *L. reuteri* CRL1098 supernatant. One representative Western Blot of three independent experiments is shown. (B) Quantitation of Western Blot of three independent experiments. (C) Determination of Lck levels in total cellular lysates and cytosolic fractions. One Western Blot of two independent experiments is shown. (D) Quantitation of Western Blot of three independent experiments. *Significantly different compared to the respective control group ($p < 0.05$, one way ANOVA test). #Significantly different compared to the raft control group ($p < 0.05$, one way ANOVA test).

10 kDa or lower 3 kDa (Fig. 6B). These results allow us to assume that the compound present in Lr-S, responsible for the reduction of TNF-α production by PBMC, has a peptide moiety component. By using a C8 column, this peptide was isolated by RP-HPLC, confirming the maintenance of the capacity of the isolated peptide to reduce the TNF-α production by control and disrupted-rafts PBMC (Fig. 6C). By mass
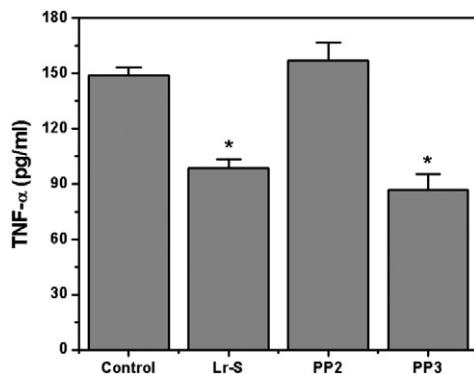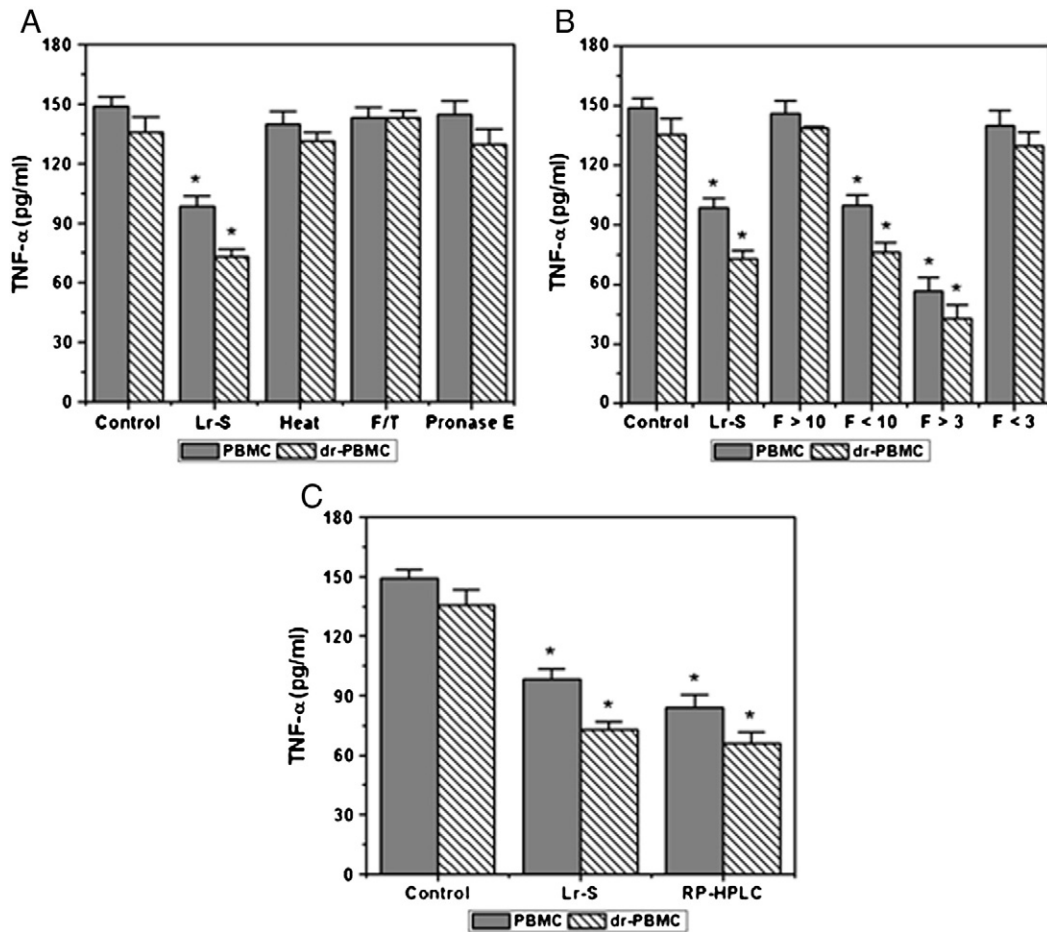
spectrometry it was identified as a 5785 Da peptide, and its sequence analysis is currently in progress.

4. Discussion

The genomics era provided novel opportunities for the characterization of bacterial probiotic effector molecules that elicit specific responses in the intestinal system. In addition, nutrigenomic analyses of the response to LAB have unravelled the signaling and immune response pathways that are modulated by probiotic bacteria. Together, genomic approaches and nutrigenomic analyses have identified several bacterial factors that are involved in modulation of the immune system [20]. During the last decade, a substantial body of scientific evidence has accumulated suggesting that certain surface-associated and extracellular components produced by probiotic LAB could be responsible for some of their mechanisms of action. The bacterial components that would be able to directly interact with the host mucosal cells include exopolysaccharides, lipoteichoic acids and surface-associated and extracellular proteins [21]. Extracellular proteins include proteins that are actively transported to the bacterial surroundings through the cytoplasmic membrane, as well as those that are simply shed from the bacterial surface. Compared to the other bacterial components, the interactive ability of extracellular proteins/peptides has been less extensively studied.

In this study we demonstrated that Lr-S exerted an immunomodulatory effect by reducing TNF-α production in PBMC. Our results also showed that soluble factors produced by *L. reuteri* CRL1098 are responsible for the reduction of TNF-α production. This contact-

Fig. 5. Effect of *L. reuteri* CRL1098 on TNF-α production by PBMC treated with Lck inhibitor. PBMC were pre-treated with 10 μM PP2 and PP3 at 37 °C and 5% CO_2 for 30 min, prior to the incubation with *L. reuteri* CRL1098 supernatant (Lr-S). TNF-α production was measured in culture supernatants by ELISA. Each value represents the mean ± SD obtained from at least three different experiments. *Significantly different compared to the control PBMC ($p < 0.05$, one way ANOVA test).

Fig. 6. Characterization of *L. reuteri* CRL 1098 supernatant (Lr-S). (A) Lr-S was submitted to heating, freezing and thawing (F/T), and Pronase E treatments prior to incubation with control (PBMC) and disrupted-rafts PBMC (dr-PBMC). TNF-α production was measured in culture supernatants by ELISA. (B) Lr-S was filtered/concentrated through 3 and 10 kDa Amicon membranes. The different fractions (>10 kDa, <10 kDa, >3 kDa and <3 kDa) were incubated with control (PBMC) and disrupted-rafts PBMC (dr-PBMC). TNF-α production was measured in culture supernatants by ELISA. (C) Fraction recovered from RP-HPLC was incubated with control (PBMC) and disrupted-rafts PBMC (dr-PBMC). TNF-α production was measured in culture supernatants by ELISA. Each value represents the mean ± SD obtained from at least three different experiments. *Significantly different compared to the respective control group ($p < 0.05$, one way ANOVA test).

independent modulation of host innate immunity by soluble factor(s) produced by lactobacilli was previously observed in human mucosal explant incubated with *Lactobacillus casei* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [22].

Previous studies reported that lipid rafts mediate activation of host cell signaling in response to attaching–effacing pathogenic bacteria [23]. Many bacteria enter to cells via lipid rafts and lipid rafts disruption disables some steps related to bacterial invasion, impairing the process [11]. While the field of pathogen–lipid raft interaction is rapidly advancing and giving rise to new possibilities for the treatment of diseases, the potential role of these microdomains in the interaction between host cells and non-pathogenic bacteria has not been investigated. To our knowledge, the first report suggesting interactions of non-pathogenic bacteria with lipid rafts was developed in our laboratory [16]. The determination of lipid rafts participation in the mechanism of interaction between probiotic bacteria and host cells would provide a better understanding of the molecular mechanism at the initial step of cells response to the bacteria. Therefore, we studied whether lipid rafts play an important role in the immuno-modulatory effect of Lr-S on PBMC. One strategy for determining the involvement of lipid rafts in signal processes is the treatment of cells with methyl-β-cyclodextrin, which depletes cholesterol from membranes. This cholesterol depletion disrupts lipid rafts domains and affects some cellular responses [24]. In this work, disrupted-rafts PBMC incubated with Lr-S showed an enhanced reduction of TNF-α levels

suggesting the involvement of lipid rafts in the decrease of TNF-α production exerted by *L. reuteri* CRL1098.

Lipid rafts spatially restrict the activation of signaling molecules that govern critical aspects of cellular activation and migration. Upon activation many signaling and stimulatory proteins are recruited to or modified in lipid rafts in order to transmit efficient intracellular signals [25]. A number of proteins have been shown to be constitutively associated with lipid raft domains on T cells, including flotillin proteins and Src-family kinases [14,15]. In this study we found that Lr-S did not modify the localization or the enrichment of Flotillin-1 in lipid rafts. On the contrary, Lr-S induced a reduction of Lck content in these microdomains. Moreover, treatment of PBMC with a Lck inhibitor blocked the effect of *L. reuteri* on TNF-α production. Several studies demonstrated that cells express a dizzying array of receptors and transmembrane proteins that transduce and integrate a huge amount of information about the environment that allows the cells to mount effective responses. The mobilization or activation of many proteins in different cells depend on the effector and the type of cells: Src family tyrosine kinases have been shown to play key roles in processes as different as oxidants-mediated signal transduction in lymphocytes and macrophages [26] adhering process of enterovirulent *E. coli* [27] and the response of innate immune cells to growth factors, extracellular matrix proteins, cytokines and pathogens [28].

Lck is a tyrosine kinase of the Src family involved in several cellular responses such as proliferation and differentiation. The role of Lck

in triggering downstream signaling pathways has been previously described for different protein stimuli that lead to modulation of cytokines production. The inactivation of proteins from the Src family, like Lck, Hck and Lyn, induces a reduction of pro-inflammatory cytokines like IL-2 in murine lymphocytes [29] and TNF-α in human macrophages [30]. Therefore, our results strongly suggest that Lck plays a role in the decreased TNF-α production induced by *L. reuteri* CRL1098.

Bacterial superantigens and viral proteins are able to improve TNF-α production by PBMC through the activation of ERK MAPK pathway [31–33]. Moreover, active compounds from plants are able to downregulate TNF-α expression in PBMC and this effect has been associated to an inhibitory effect on ERK phosphorylation [33]. On the contrary, Morjaira et al. [34] showed that imidazoquinoxalines are able to reduce TNF-α production in PBMC by activating p38 MAPK and inhibiting the PI3K pathway. Therefore, p38 but not ERK MAPK pathway could be involved in the downregulation of TNF-α production in PBMC induced by Lr-S. More detailed studies are necessary to determine the precise participation of MAPK pathways in the immunomodulatory effect of Lr-S.

Finally, in addition to the modulatory effect of Lr-S on PBMC, in the present work, we wanted to extend the knowledge of the chemical nature of the soluble metabolite(s) responsible for the effect of *L. reuteri* CRL1098. Our results allow us to assume that among the metabolites secreted by *L. reuteri* CRL1098, a peptide of 5785 Da would be responsible for the reduction of TNF-α production by PBMC.

Recent findings suggest that the crosstalk between the commensal microbiota and the local immune system is partially elicited through soluble factors and not exclusively through direct cell contact. Moreover, several *in vitro* studies demonstrated that peptides secreted by probiotic strains are able to functionally modulate the immune system. It has been established that a secreted peptide produced by *L. plantarum* BMCM12, resistant to intestinal proteolysis and found in the human colonic microenvironment, is capable of modulating phenotype and function of human dendritic cells [35]. Two isolated proteins (p40 and p75) secreted by *L. rhamnosus* GG were found effective in preventing intestinal epithelial cells lines from cytokine-induced apoptosis by regulating signaling cascade through Akt activation [36]. In addition, *L. plantarum* 10hk2-specific extracellular protein metabolites induce anti-inflammatory responses such as downregulation of TNF-α release in RAW 264.7 cells cultured with LPS [4]. Similar results highlighting the role of bacterial-derived products have recently reported that peptides secreted by probiotics can modulate the immune system *in vivo*. In this sense, it was demonstrated that soluble factors released by the probiotic bacteria *Bifidobacterium breve* C50 can downregulate the production of inflammatory cytokines by immune cells with these factors maintaining their inhibitory activity after crossing an epithelial barrier [5]. These soluble factors produced by the C50 strain can effectively dampen intestinal inflammation in a mouse model of colitis by targeting both epithelial and local dendritic cells [37]. Our present results encompasses these known effects of similar bioactive proteins present in Lr-S and moreover, gain some mechanistic insight about the immunomodulatory effect of probiotic's secreted peptides by demonstrating lipid rafts involvement. Our future experimental work will focus on structural identification and detailed immunomodulatory effect mapping of the isolated peptide.

In conclusion, this work constitutes the first report shedding light on the evidence about the involvement of lipid rafts in the modulation of cytokine production induced by soluble factors of a beneficial LAB like *L. reuteri* CRL1098. The knowledge of lipid rafts participation in the response induced by *L. reuteri* CRL1098 opens new possibilities for studying the mechanisms involved in the interaction of LAB with the host immune cells. In addition, this probiotic-derived peptide could be useful in the treatment of inflammatory diseases considering the fact that numerous studies have shown that reducing the levels of TNF-α through the use of anti-TNF-α antibodies or soluble TNF receptors is a safe and efficacious treatment for inflammatory diseases such as rheumatoid arthritis. Our laboratory is actively working to fully characterize and purify the peptide responsible for the modulatory effect of *L. reuteri* CRL1098 in order to study the capacity of these new potential pharmabiotic to modulate unproductive inflammation *in vivo*.

Acknowledgments

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