Stimulation of Innate Immune Cells Induced by Probiotics: Participation of Toll-Like Receptors

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Abstract

Objective: The present work aimed to study the functionality of macrophages from different locations (peritoneum, spleen and Peyer´s patches) when they were stimulated with probiotics microorganisms: *Lactobacillus casei* CRL 431 and *Lactobacillus paracasei* CNCM I-1518 or a Probiotic Fermented Milk (PFM) through Toll-Like Receptors (TLRs), prior challenged with agonists or antagonists of TLRs.

Methods: BALB/c mice received in the drinking water, the probiotic bacteria (*L. casei* CRL 431 and *L. paracasei* CNCM I-1518) or the PFM. We focused our investigation mainly on the phagocytic activity of macrophages from peritoneum, spleen and Peyer's patches and cytokine production were evaluated prior challenged with TLR2 and TLR4 agonists or antagonists. The microbicidal activity of macrophages and against an infection with *Salmonella typhimurium* was also studied. To assess the role of TLR in probiotic stimulation, we evaluated the phagocytic activity, cytokine production and Immunoglobin G (IgG) anti-OVA in C57BL/6 knockout mice to MyD88, TLR2 and TLR4.

Results: In BALB/c mice, the best effect in the phagocytosis assay was obtained with the probiotic bacteria *L. casei* CRL 431, this effect was reinforced with TLR2 agonist. The production of different cytokines (IL-10 and IL-6) was improved with the probiotic treatments and this production was ameliorated with TLRs agonists. The antimicrobial activity was increased with *L. casei* CRL 431 and *L. paracasei* CNCM I-1518, TLR2 and TLR4 antagonists had a negative effect on microbicidal activity. The administration of probiotic bacteria or PFM improved the host response against *S. typhimurium* controlling the infection during the first hours post-infection. In C57BL/6 knockout mice, phagocytic activity was significantly diminished in comparison to wild type mice and the probiotic bacteria or PFM administration was not able to improve this activity. The IL-10 production was increased at a concentration of 10^8 cells/ml of *L. casei* CRL 431 in TLR2$^{-/-}$ and TLR4$^{-/-}$, but not in MyD88$^{-/-}$ mice. The administration of probiotic bacteria or PFM did not play a stimulating effect in the systemic immune response against to OVA antigen in knockout mice.

Conclusions: Probiotics modulate the different signaling pathways of innate immune cells through the TLRs. The macrophages activation depends on location of them and that different probiotic strains of *Lactobacilli* can evoke different intensity of responses. The data suggest that probiotic not only promote a major expression of TLRs but also use these receptors via the innate immune cells as macrophages to stimulate and modulate the immune response.

Introduction

Innate immune cells are often described as the 'sentinel of the immune system' because they are among the first cell types to react in the host, against pathogens, tumor, or differents injuries [1].

The cells that play a critical role in initiating the innate immune response are the macrophages and dendritic cells [2]. Macrophages are resident phagocytic cells in lymphoid and non-lymphoid tissues and they are involved in steady-state tissue homeostasis, via the clearance of apoptotic cells and immunity [3-5]. Tissue-resident macrophages are best known for their role as immune sentinels in the frontline of tissue defense where they are discretely positioned and transcriptionally programmed for the encounter with pathogens or

environmental challenges [4]. The phenotype of resident macrophages in tissues is determined by the tissue microenvironment, the extracellular matrix, secretory products and surface molecules of neighboring cells.

Macrophages express a wide array of Pattern Recognition Receptors (PRR) to recognize and respond to Microbe-Associated Molecular Patterns (MAMPs). This recognition induces a signaling cascade that can result in the production of cytokines, chemokines, and other effector molecules activating the innate immune response in the host [6]. The Toll-Like Receptors (TLRs) family, which is the best PRR characterized, includes TLR1, 2, 4, 5, 6 and 11 recognize mainly microbial membrane components and are expressed on the cell surface, and TLR3, 7, 8 and 9 that recognize nucleic acids of bacterial or viral origin, are expressed in intracellular compartments [7].

Lactobacilli are able to modulate immune response of the host by interaction with the immune cells and the intestinal epithelium. There are many evidence concerning to the role of probiotics, especially for Lactic Acid Bacteria (LAB), in the maintenance of health or in the prevention of disease [8]. Probiotics are defined as "live microorganisms which, when administered in adequate amounts confer a health benefit to the host" [9]. Studies have shown that different strains of *Lactobacilli* can evoke different responses in the host [10], therefore the results from one strain cannot be generalized to others.

The aim of this work was to study by *in vitro* and *ex vivo* assays, the participation of TLRs in macrophages when they are stimulated by different probiotics bacteria or Probiotic Fermented Milk. The effect from macrophages of Peyer's patches and in distant sites from intestine such as peritoneum and spleen were studied. We focused on the role of TLR2 and 4, present in the surface of macrophages, that are involved in innate immunity and the way by which the oral administration of probiotics bacteria or Probiotic Fermented Milk through of these TLR mediate activation of these cells measuring the phagocytic and microbicidal activity. The previous studies were confirmed using knockout mice to MyD88$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$. We also analyzed by *in vivo* assays the antimicrobicidal activity against an intraperitoneal infection with *Salmonella typhimurium* prior stimulation with probiotic bacteria or Probiotic Fermented Milk.

Methods

Mice

BALB/c mice weighing 25 to 30 g (6 weeks of age) were obtained from the inbred colony maintained at Institute of Microbiology of Facultad de Bioquímica, Química y Farmacia de la Universidad Nacional de Tucumán. The animals were fed on balanced rodent food and water ad libitum. Wild-type (WT) C57BL/6 mice were purchased from Janvier (France). MyD88$^{-/-}$, TLR4$^{-/-}$ and TLR2$^{-/-}$ mice were kindly provided by Dr. S. Akira, Japan, crossed in the C57BL/6 background and bred in the animal facilities at the Hôpital Necker of Paris, France, under specific pathogen-free conditions. All experiments have been conducted in accordance with the European Union Council Directives (86/609/EEC) and with the institutional guidelines (INSERM: Institut National de la Santé et de la Recherche Médicale). The animal facility is accredited by an agreement delivered by the Prefecture de Police of Paris, France and for the guidelines from Universidad Nacional de Tucumán, Argentina.

Bacterial Strains and Culture Conditions

Lactobacillus casei CRL 431 and *Lactobacillus paracasei* CNCM I-1518 were obtained from the CERELA culture collection (San Miguel de Tucumán, Argentina). The *Samonella enterica serovar typhimurium* (*S. typhimurium*) used in this study was obtained from the Bacteriology Department of the Hospital del Niño Jesús (San Miguel de Tucumán, Argentina). *L. casei* and *L. paracasei* were cultured in a sterile Man, Ragosa and Sharpe (MRS) broth (Britania, Buenos Aires, Argentina) and *S. typhimurium* was cultured in a sterile Brain Heart Infusion (BHI) broth (Britania, Buenos Aires, Argentina).

Probiotic Fermented Milk (PFM)

Commercial Probiotic Fermented Milk (PFM) containing as starters *Lactobacillus delbrueckii* subsp. *bulgaricus* (10^8 CFU/ml) and *Streptococcus thermophilus* (10^8 CFU/ml) and probiotic strain *Lactobacillus paracasei* CNCM I-1518 (10^8 CFU/ml) was used.

L. casei and Probiotic Fermented Milk Administration

For *ex vivo* assays, the mice were housed in individual boxes of three mice each one and given a viable *Lactobacilli* suspension in the drinking water, *L. casei* CRL 431 was administered for 7 consecutive days and *L. paracasei* CNCM I-1518 or Probiotic Fermented Milk (PFM) for 5 consecutive days, due to these time were the optimal period of administration for the probiotic bacteria and PFM to activate the intestinal immune system in healthy animals, previously determined in our laboratory [8,11]. The bacterial suspensions were prepared from overnight cultures grown at 37°C in 5 ml of MRS broth. The cells were harvested by centrifugation at 5,000 g for 10 min, washed three times with fresh Phosphate Saline Solution (PBS) and then re-suspended in 5 ml of sterile 10% (wt/vol) local commercial skim milk powder (Svelty Figura, Nestlé, Argentina). The bacterial suspensions were diluted 1:30 in water and administered *ad libitum* to the mice. The final concentration of probiotic bacteria was $2 \pm 1 \times 10^8$ CFU/ml. The PFM was also administered *ad libitum* to the mice.

Ex Vivo Phagocytosis Assay of Peritoneal, Spleen and Peyer's Patches Macrophages

Peritoneal, spleen and Peyer's patches macrophages from BALB/c mice treated with probiotic bacteria or PFM, were obtained according to a previously described method in our laboratory [12,13], and the concentration was adjusted at 1×10^6 cells/ml. The macrophages were stimulated with 20 µl Pam3CSK4 (1 µg/ml) (Synthetic triacylated lipoprotein-TLR1/2 ligand - InvivoGen, San Diego, CA, USA), MAb mTLR2 (10 µg/ml) (Purified monoclonal antibody to mouse TLR2 - InvivoGen), LPS-EB Ultrapure (10 µg/ml) (Ultrapure lipopolysaccharide from E. coli 0111:B4 strain-TLR4 ligand - InvivoGen) and LPS-RS Ultrapure (10 µg/ml) (Ultrapure lipopolysaccharide from Rhodobacter sphaeroides -TLR4 antagonist – InvivoGen) (2 h; 37°C; 5% CO$_2$).

Phagocytosis assay was performed using a *Saccharomyces cerevisiae* suspension at a concentration of 10^7 cells/ml. Opsonized yeast in mouse autologous serum (10%) were added to 200 µl of macrophage suspension. The mixture was incubated for 30 min at 37°C. The percentage of phagocytosis was expressed as the percentage of phagocyting macrophages in 100 cells counted in an optical microscope.

In Vitro Assays for Cytokine Determination

The cytokines IL-6 and IL-10 production by peritoneal, spleen and Peyer's patches macrophages were determined by *in vitro* assay. Peritoneal, spleen and Peyer's patches macrophages from untreated BALB/c mice were isolated on RPMI-1640 medium (Sigma, St. Louis, Mo) and adherent in TC-Plates (6 wells, sterile with LID Cellstar Greiner bio-one) (1 h; 37°C; 5% CO_2) and the final concentration was adjusted at 1×10^6 cells/ml/wells. TLR2 and TLR4 agonist and antagonist 20 µl (1, 10 or 10, 10 µg/ml, respectively) (InvivoGen, San Diego, CA, USA) were added to the cells cultures (2 h; 37°C; 5% CO_2). Overnight cultures of *L. casei* CRL 431 and *L. paracasei* CNCM I-1518 were centrifuged (5,000 rpm; 10 min), washed three times with PBS sterile, and 20 µl was added to the macrophages culture to final concentrations of 10^8 CFU/ml and 20 µl of PFM was added directly to the culture. They were incubated (12 h; 37°C; 5% CO_2). The supernatants of culture media were recovered for cytokine determination by ELISA test according the manufacturer's instructions (BD OptEIA BD bioscience, San Diego, USA).

Ex Vivo Microbicidal Activity of Peritoneal and Spleen Macrophages

After 7 days of administration of *L. casei* CRL 431 and 5 days of administration of *L. paracasei* CNCM I-1518 and PFM respectively, the peritoneal and spleen macrophages from BALB/c mice were cultured in RPMI-1640 medium (Sigma, St. Louis, Mo) and incubated in TC-Plates (6 wells, sterile with LID Cellstar Greiner bio-one) (1 h; 37°C; 5% CO_2), the final concentration was adjusted at 1×10^6 cells/ml/ wells. The macrophages were incubated with 20 µl of TLR2 or TLR4 antagonists (10 and 10 µg/ml, respectively) (InvivoGen, San Diego, CA, USA) (2 h; 37°C; 5% CO_2) and then were infected with 1 ml of 1×10^7 CFU of *S. typhimurium*. The bacterium/macrophage ratio was 10:1. After 30 min of incubation at 37°C, extracellular bacteria were gently removed by washing with PBS and then RPMI medium containing 100 µg/ml of gentamicin was added and incubated for 60 min. Finally, the cells were lysed with 1 ml of Triton X-100 1% in RPMI. Serial dilutions of the suspension of cells lysed were plated on SS agar (Salmonella - Shigella agar, Britania, Buenos Aires Argentina). The counting forming units (CFU) of *S. typhimurium* were determinate after incubation for 24 h at 37°C.

In Vivo Challenge with *Salmonella typhimurium*

BALB/c mice were fed with *L. casei* CRL 431 (7 consecutive days), *L. paracasei* CNCM I-1518 (5 consecutive days) or PFM (5 consecutive days). After that, mice were infected intraperitoneally with 100 µl of a suspension of *Salmonella typhimurium* (10^6 CFU/ml). The mice were sacrificed by cervical dislocation. The animal protocols were according to the Guide for the Care and Use of Laboratory Animals – National Research Council, 1996. All animal protocols were pre-approved by the Ethical Committee of Universidad Nacional de Tucumán and all experiments comply with the current laws of Argentina. Liver and spleen were aseptically removed and placed into sterile tubes containing 5 ml of peptone water (0.1%). The samples were immediately homogenized and serial dilutions were made and spread onto the surface of SS agar (Salmonella – Shigella agar, Britania, Buenos Aires, Argentina). The counting of bacterial cells in spleen and liver were checked in the following post-infection time intervals: 18, 24, 30, 48 and 72 hours.

Ex Vivo Phagocytosis Assay of Peritoneal Macrophages in Knockout Mice

C57BL/6 mice six-weeks-old were divided into 4 groups: wild type, MyD88$^{-/-}$, TLR4$^{-/-}$ and TLR2$^{-/-}$ group. Each group was divided into 3 subgroups according to the dietary supplement: water, *L. casei* CRL 431 suspension for 7 days, or PFM for 5 days. At the end of the feeding period, the animals were sacrificed. Peritoneal macrophages, were isolated and the concentration were adjusted at 1×10^6 cells/ml. Phagocytosis assay was performed using a *Saccharomyces cerevisiae* suspension at a concentration of 1×10^7 cells/ml as was previously described.

IL-10 Production by Peritoneal Macrophages from Knockout Mice

Peritoneal macrophages from untreated wild type, MyD88$^{-/-}$, TLR4$^{-/-}$ and TLR2$^{-/-}$ C57BL/6 mice were isolated on RPMI-1640 medium (Sigma-Aldrich, St. Louis, Mo, USA) supplemented with antibiotics and 10% fetal calf serum (Invitrogen, Cergy-Pontoise, France). A volume of 500 µl (1×10^6 cells/ml) was plated per well into 24-wells plates. Macrophages were then cultured with different concentrations (10^9, 5×10^8, 10^8, 5×10^7, 10^7, 5×10^6, 10^6 and 5×10^5) of the probiotic bacterium *L. casei* CRL 431 for 24 hrs at 37°C. Culture supernatants were harvested and stored at -80°C until cytokines were measured by ELISA test according the manufacturer's instructions (R&D systems, Lille, France).

Detection of Anti OVA IgG in Serum

A wild type and three knockout mice (MyD88$^{-/-}$, TLR4$^{-/-}$ and TLR2$^{-/-}$ C57BL/6) were used in this study. For each class of mouse, three groups were used according to the dietary supplement: water, *L. casei* CRL 431 suspension for 7 days or PFM for 5 days.

At the end of the feeding period, all the animals were injected subcutaneously with 100 µl three times every 48 hours with 15 µg of chicken egg albumin (OVA) (Sigma-Aldrich, St. Louis, Mo, USA) in PBS plus adjuvant complete Freund (Difco Laboratories, Detroit, Michigan, USA) (1:1). The serum samples were collected 20 days after of the immunization and storage at -80°C until specific anti OVA IgG determination by ELISA test according the manufacturer's instructions (R&D systems, Lille, France).

Statistics

For all the studies, each group assayed was of 3 mice. The results are showed as the means of three independent trials with their respective SEM (standard error of the mean) from 9 mice. Statistical analysis was performed using MINITAB 15 software (Minitab, Inc.) by ANOVA general linear model followed by Tukey's post hoc test, and P≤0.05 was considered significant.

Results

Probiotic Effect on the Phagocytic Activity of Macrophages: Implication of TLRs

To evaluate the role of TLRs in macrophages activation, we determined the percentage of phagocitosis. Peritoneal macrophages activity was increased significantly after *L. casei* CRL 431 and PFM stimulation respect to the untreated group and it also showed an

increase when the cells were previously confronted with TLR2 agonist. The same behavior was obtained when antagonized the TLR2, where the phagocytic activity was increased only in macrophages from mice that were treated with *L. casei* CRL 431. However, the phagocytic activity decreased when the macrophages were faced to TLR4 agonist or antagonist respect to the control group and this activity does not improve in the cells that were previously stimulated with *L. casei* CRL 431 or PFM (Figure 1A).
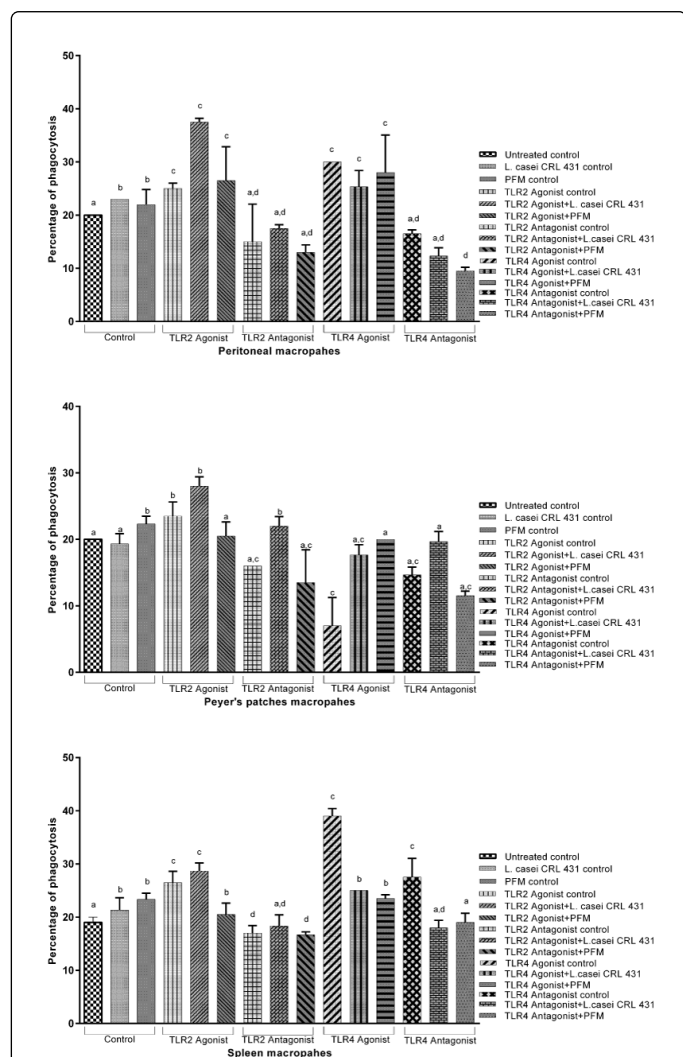

Figure 1: Phagocytic activity of macrophages isolated from peritoneum, Peyer's patches and spleen. Effects of TLR2 and TLR4 agonist and antagonist on phagocytic activity of macrophages from BALB/c mice stimulated previously with *Lactobacillus casei* CRL 431, 7 consecutive days of administration and probiotic fermented milk, 5 consecutive days of administration. (A) Peritoneal macrophages. (B) Peyer's patches macrophages. (C) Spleen macrophages. [a,b,c,d]Means values for peritoneum, Peyer's patches and spleen macrophages without a common letter differ significantly (P<0.05). The error bars indicate standard deviations for 3 independent determinations per mouse.

The assay performed on Peyer's patches showed a significant increase of the activity of macrophages stimulated with PFM

compared to untreated group. There was an increased in the phagocytic activity in those macrophages stimulated with *L. casei* CRL 431 and then confronted with TLR2 and TLR4 agonists and antagonists, respect to the controls group. Regard to PFM, the phagocytic activity was increased only when the macrophages were faced to TLR4 agonist, compared with the control (Figure 1B).

The analysis of the phagocytic activity on spleen macrophages showed that both *L. casei* CRL 431 and the PFM supplementation induced significant increases in this activity compared to the untreated group. Stimulation with probiotic bacteria and then confronted with TLR2 agonist or antagonist showed slight increase compared to their control group, whereas stimulation with PFM showed a decrease in the phagocytic activity. On the other hand, stimulation with TLR4 agonist or antagonist showed a decrease in the activity compared to their control group (Figure 1C).

IL-10 and IL-6 Determination

The profile of IL-10 production by peritoneal macrophages showed a significant increase when these cells were stimulated with *L. casei* CRL 431 or PFM compared to untreated control, and the same pattern were obtained with TLRs agonists and antagonists. Whereas, the stimulation with *L. paracasei* CNCM I-1518 showed not significant increase (Figure 2A).
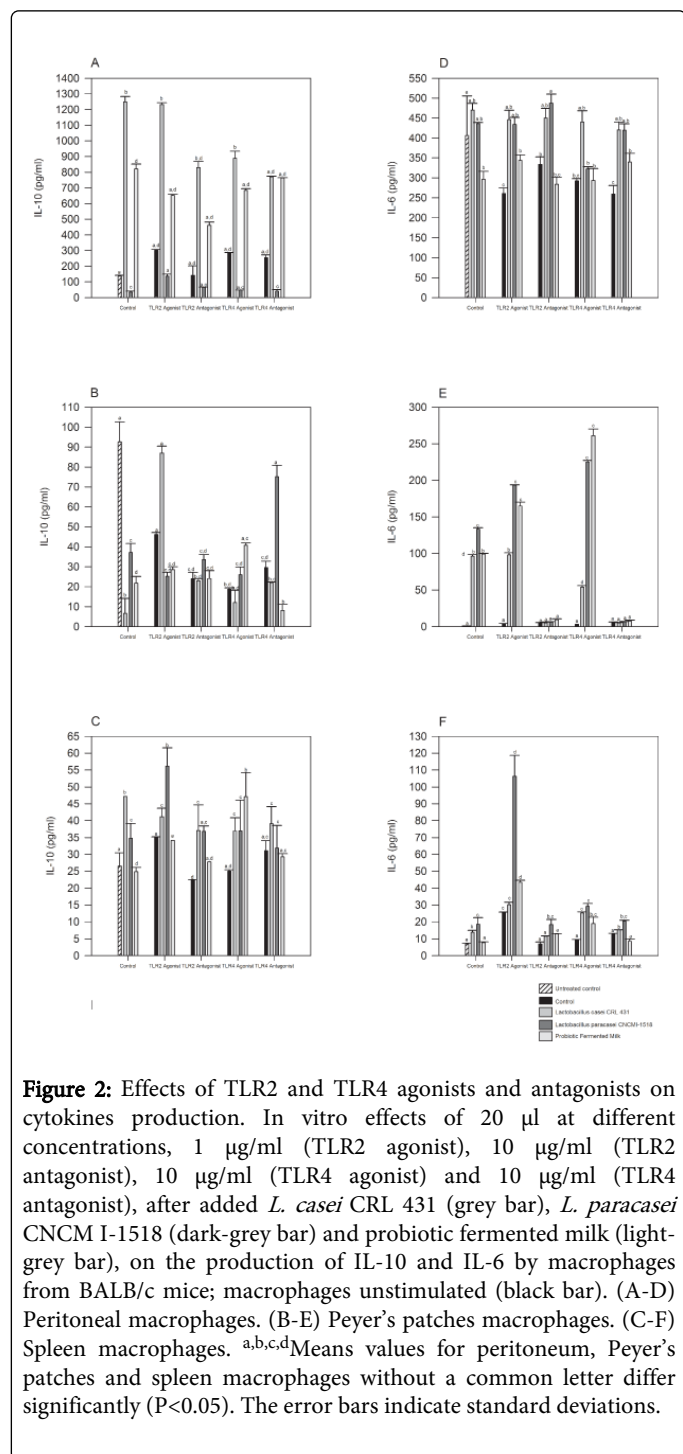
The IL-10 production from Peyer's patches showed a significant decrease when the macrophages were stimulated with *L. casei* CRL 431, *L. paracasei* CNCM I-1518 or PFM compared to the untreated control. We observed similar values to the untreated control, when the macrophages were confronted with TLR2 agonist for *L. casei* CRL 431, while there was an increase of IL-10 production from macrophages faced to TLR2 and TLR4 antagonists when the macrophages were stimulated with *L. paracasei* CNCM I-1518 compared to their respective controls. As regard to PFM, IL-10 production was increased only when the macrophages were faced previously to TLR4 agonist (Figure 2B).

Spleen macrophages showed a significant increase of IL-10 production when the cells were stimulated with *L. casei* CRL 431 or *L. paracasei* CNCM I-1518 compared to the untreated control, while the stimulated with PFM, showed similar result to the control. When the macrophages were stimulated with probiotic bacteria and confronted with TLRs agonists, there was an increased release of IL-10 compared to their respective control group, while stimulation with PFM, only showed increases when was faced to TLR4 agonist. For to TLR4 antagonist we did not observed differences respect the control group (Figure 2C).

The IL-6 productions by peritoneal macrophages showed that this cytokine was not increased in macrophages stimulated with probiotic bacteria and PFM compared to untreated control. Generally, there was an increased production of the IL-6 when the macrophages were stimulated with *L. casei* CRL 431 or *L. paracasei* CNCM I-1518 previous showdown with TLRs agonists or antagonists respect to the control, whereas PFM showed an increase when macrophages were faced to TLR2 agonist and TLR4 antagonist, compared to the control groups (Figure 2D).

Peyer's patches macrophages showed an increased level of IL-6 production when these cells were stimulated with *L. casei* CRL 431, *L. paracasei* CNCM I-1518 or PFM compared to the untreated control. When the macrophages were stimulated with probiotic bacteria or PFM in addition to TLRs agonist, the cytokine production showed

significant increases compared to the control groups. On the other hand, when the macrophages were confronted with TLRs antagonist the probiotic bacteria or PFM, showed a decrease in the IL-6 production compared to the control groups (Figure 2E).

Figure 2: Effects of TLR2 and TLR4 agonists and antagonists on cytokines production. In vitro effects of 20 μl at different concentrations, 1 μg/ml (TLR2 agonist), 10 μg/ml (TLR2 antagonist), 10 μg/ml (TLR4 agonist) and 10 μg/ml (TLR4 antagonist), after added *L. casei* CRL 431 (grey bar), *L. paracasei* CNCM I-1518 (dark-grey bar) and probiotic fermented milk (light-grey bar), on the production of IL-10 and IL-6 by macrophages from BALB/c mice; macrophages unstimulated (black bar). (A-D) Peritoneal macrophages. (B-E) Peyer's patches macrophages. (C-F) Spleen macrophages. [a,b,c,d]Means values for peritoneum, Peyer's patches and spleen macrophages without a common letter differ significantly (P<0.05). The error bars indicate standard deviations.
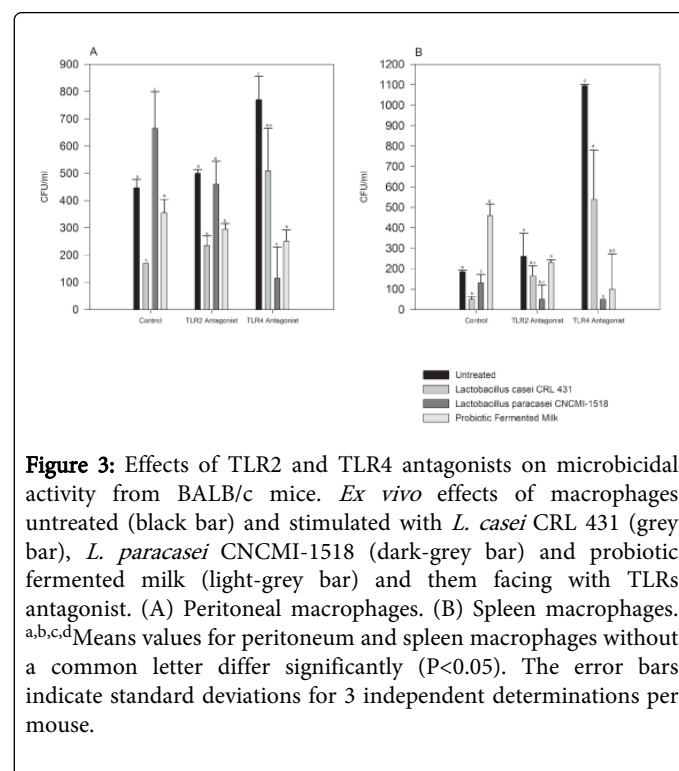
The IL-6 production in spleen macrophages showed a significant increase when the cells were stimulated with probiotic bacteria *L. casei* CRL 431 and mainly with *L. paracasei* CNCM I-1518 compared to the untreated control, being these increases more significant for the TLRs

agonists. This cytokine also showed an increase when the macrophages were stimulated with *L. casei* CRL 431 or *L. paracasei* CNCM I-1518 for TLRs antagonist; however these values were lower than those obtained with the TLR agonist (Figure 2F).

Ex Vivo Assays of Microbicidal Activity of Peritoneals and Spleen Macrophages

The microbicidal activity of peritoneal macrophages decreased when the TLRs antagonist were added to the cells culture. When the animals were fed with a *L. casei* CRL 431 or PFM, the microbicidal activity increased compared to the untreated control (P<0.05). When the animals were fed with *L. casei* CRL 431 or PFM and challenged with antagonists of TLRs, an increased microbicidal activity was showed, in comparison to the untreated control group. *L. paracasei* CNCM I-1518 showed an increase in microbicidal activity when the cells were facing to TLR4 antagonist (Figure 3A).

Figure 3: Effects of TLR2 and TLR4 antagonists on microbicidal activity from BALB/c mice. *Ex vivo* effects of macrophages untreated (black bar) and stimulated with *L. casei* CRL 431 (grey bar), *L. paracasei* CNCMI-1518 (dark-grey bar) and probiotic fermented milk (light-grey bar) and them facing with TLRs antagonist. (A) Peritoneal macrophages. (B) Spleen macrophages. [a,b,c,d]Means values for peritoneum and spleen macrophages without a common letter differ significantly (P<0.05). The error bars indicate standard deviations for 3 independent determinations per mouse.

Spleen macrophages showed that animals stimulated with *L. casei* CRL 431 had higher antimicrobial activity than the untreated control (P<0.05), but when the cells were blocked with the TLRs antagonist, this activity decreased compared to the control group. In the case of animals treated with *L. paracasei* CNCM I-1518 a slight increase of the microbicidal activity was shown compared to normal control but when the cells were antagonized for TLRs, macrophages showed an increase in the microbicidal activity compared to control group. The PFM showed less microbicidal activity than the untreated animals, but this activity increased when cells were antagonized for TLR2 and TLR4, respectively (Figure 3B).

In Vivo Antimicrobial Activity Induced by Probiotic Against an Intraperitoneal Infection with *S. typhimurium*

We analyzed if the macrophages activity induced by treatment with probiotic bacteria or PFM was effective against an intraperitoneal infection with *Salmonella typhimurium*.

We observed in both liver and spleen tissues that treatment with the probiotics assayed and the PFM controlled the bacterial counts of *S. typhimurium* during the first 24 to 30 hours, after the infection, then they could not control the infection and the CFU values were similar to the untreated infection animals, with an increased translocation of *S. typhimurium* to the liver and spleen (Figures 4A, 4B).
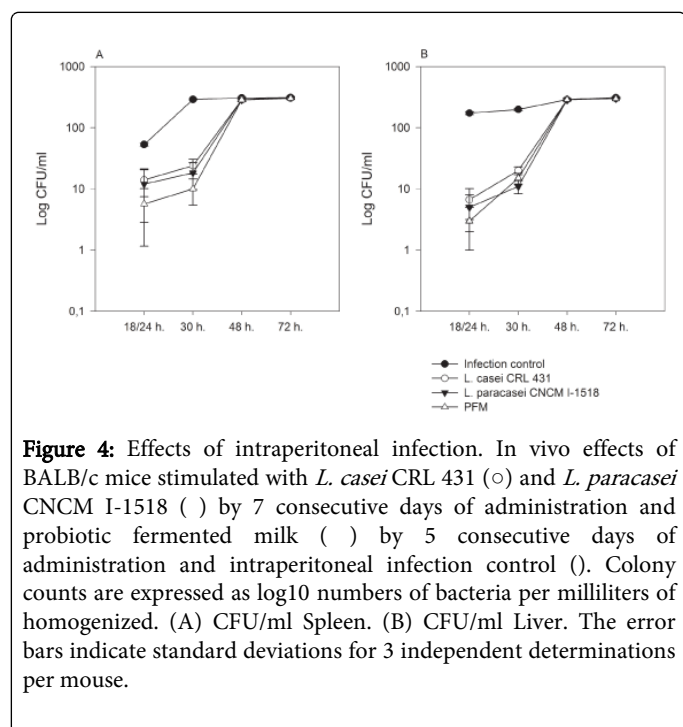
Figure 4: Effects of intraperitoneal infection. In vivo effects of BALB/c mice stimulated with *L. casei* CRL 431 (○) and *L. paracasei* CNCM I-1518 ( ) by 7 consecutive days of administration and probiotic fermented milk ( ) by 5 consecutive days of administration and intraperitoneal infection control (). Colony counts are expressed as log10 numbers of bacteria per milliliters of homogenized. (A) CFU/ml Spleen. (B) CFU/ml Liver. The error bars indicate standard deviations for 3 independent determinations per mouse.

Validation of the Previous Results Studies in Knockout Mice

Ex Vivo **phagocytosis assay:** The results obtained with the wild type C57BL/6 mice showed that the PFM administration induced significant increases in the phagocytic activity of peritoneal macrophages compared to the normal control wild type mice. In knockout animals (MyD88$^{-/-}$, TLR4$^{-/-}$ and TLR2$^{-/-}$ C57BL/6), the ability to phagocytose yeast was decreased to about half of the value comparatively to the wild type mice. The probiotic bacterium *L. casei* CRL 431 or the PFM administration had no effect on the activity of the peritoneal macrophages (Figure 5).

IL-10 Production from Peritoneal Macrophages Stimulated with *L. Casei* CRL 431

In vitro activation of the peritoneal macrophages showed that high and low concentrations of the probiotic bacteria were not able to induce IL-10 production. However, *L. casei* CRL 431 at a concentration of 108 cells/ml showed a significant production of IL-10 even in knockout TLR2 and TLR4 animals. However deficiency of MyD88 was decisive for the production of this cytokine from peritoneal macrophages (Figure 6).
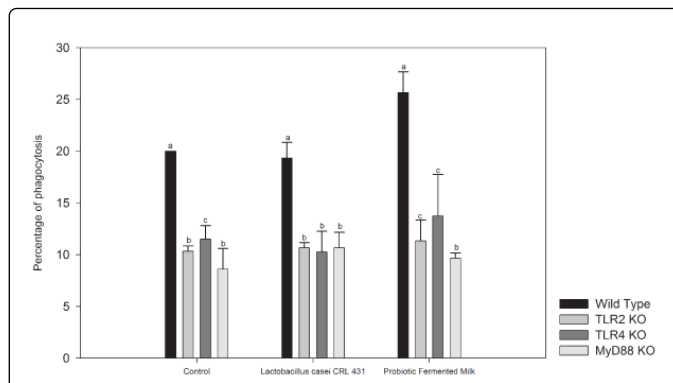

Figure 5: Phagocytic activity of peritoneal macrophages from knockout mice. Phagocytic activity of macrophages from wild type C57BL/6 (black bar), TLR2 $^{-/-}$ C57BL/6 (grey bar), TLR4$^{-/-}$ C57BL/6 (dark-grey bar), and MyD88$^{-/-}$ C57BL/6 (light-grey bar) mice stimulated previously with *Lactobacillus casei* CRL 431 by 7 consecutive days of administration and probiotic fermented milk, 5 consecutive days of administration. ^{a,b,c,d}Means values for peritoneal macrophages without a common letter differ significantly (P<0.05). The error bars indicate standard deviations for 3 independent determinations per mouse.
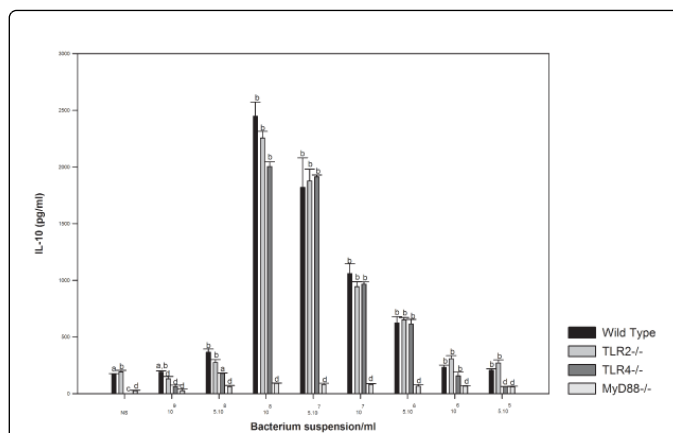

Figure 6: IL-10 production of peritoneal macrophages from knockout mice. Effects of different concentrations of *L. casei* CRL 431 on the production of IL-10 of peritoneal macrophages from wild type C57BL/6 (black bar), TLR2 $^{-/-}$ C57BL/6 (grey bar), TLR4$^{-/-}$ C57BL/6 (dark-grey bar), and MyD88$^{-/-}$ C57BL/6 (light-grey bar) mice. ^{a,b,c,d}Means values for peritoneal macrophages without a common letter differ significantly (P<0.05). The error bars indicate standard deviations.

Influence of Probiotics on the Systemic Immune Response

For the evaluation of the systemic immunity, the level of the specific-anti-OVA IgG in serum after OVA immunization was evaluated. The probiotic bacterium *L. casei* CRL 431 or the PFM administration did not show increases in the anti-OVA IgG in serum of wild type mice respect to immunized control. As regard to PFM, the levels of specific IgG showed a decrease in TLR2$^{-/-}$ and MyD8$^{-/-}$ C57BL/6 mice in comparison to the immunized control group and

respect to wild type mice; however TLR4$^{-/-}$ showed similar values to the immunized control. Whereas *L. casei* CRL 431 showed similar values to immunized control in all cases. The probiotic bacterium administration did not have a greater influence in the anti-OVA IgG levels in the knockout mice. The data suggest that TLR would not be involved in the anti-OVA response (Figure 7).
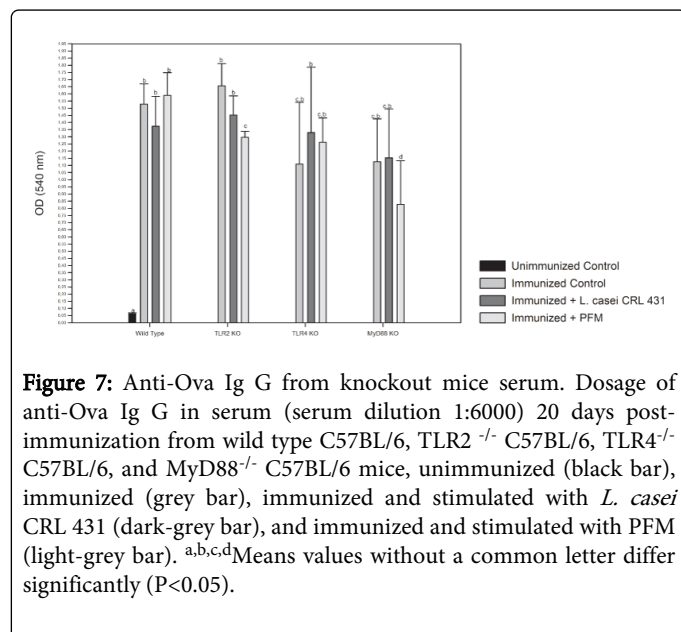

Figure 7: Anti-Ova Ig G from knockout mice serum. Dosage of anti-Ova Ig G in serum (serum dilution 1:6000) 20 days post-immunization from wild type C57BL/6, TLR2 $^{-/-}$ C57BL/6, TLR4$^{-/-}$ C57BL/6, and MyD88$^{-/-}$ C57BL/6 mice, unimmunized (black bar), immunized (grey bar), immunized and stimulated with *L. casei* CRL 431 (dark-grey bar), and immunized and stimulated with PFM (light-grey bar). [a,b,c,d]Means values without a common letter differ significantly (P<0.05).

Discussion

At the mucosal level, the innate immune response not only provides the first line of defense against pathogenic microorganisms but also provides the biological signals to evoke the adaptive immune response. The key process is the recognition of microbial agents by Pattern-Recognition Receptors (PRRs) which detect conserved microbial ligands called Pathogen-Associated Molecular Patterns (PAMPs) [14]. This receptor system allows a clear and precise immune response.

Much attention has been focused on signal mediated by TLRs. By detecting PAMPs, TLR engagement leads to adaptor molecule Myeloid Differentiation Factor 88 (MyD88) recruitment and subsequent NF-κB and MAPK-mediated transcriptional activation of a range of cytokines and chemokines that orchestrate the early host resistance to infection [15]. Moreover TLR signaling has been shown to have a protective role in the intestine [16,17]. The constitutive activation of TLR signaling in the intestinal epithelial cells is necessary to maintain the epithelial homeostasis and regulate the composition of luminal microorganisms by induction of the antimicrobial peptides and mucosal immunoglobulin A secretion. Studies performed in MyD88 deficient mice, showed more susceptibility to spontaneous intestinal infections and defects in the antimicrobial peptides production by Paneth cells [18].

Previous studies conducted in our laboratory showed that probiotic bacteria induced increases in the TLRs expression in the immune cells of lamina propria of the small intestine and in the Peyer´s patches, which seemed to suggest the involvement of these receptors, in the signaling pathways induced by probiotic microorganisms in the gut [8,19]. *In vitro* studies demonstrated that two *Lactobacillus* strains, *L. rhamnosus* GG and *L. plantarum* BFE 1685 increase the expression of TLR2 in intestinal epithelial cells line HT-29 [20]. These studies led us

to determine whether probiotic bacteria or PFM administration are able to send signals to increase the phagocytic activity of macrophages distant from the gut such as peritoneal and spleen macrophages. We observed that the best effect was obtained with the probiotic bacteria administration (Figures 1A and 1C). We evaluated the importance of the mentioned receptors, using TLR2 and TLR4 agonists and antagonists and evaluated the immune adjuvant capacity of the probiotic administration. Comparatively, for peritoneal macrophages, the probiotics assayed or PFM administration had a similar effect to that produced by TLR2 agonist, increasing the phagocytic activity of these cells. In contrast, the addition of antagonist for two TLRs studied, the phagocytic activity diminished even when the probiotic or PFM were administered to mice. These results indicate that TLRs are, not only involved in the phagocytic process of peritoneal macrophages, but also they are a pathway used by probiotic microorganisms to activate these cells. Similar results were obtained in spleen macrophages (Figures 1A and 1C).

The intestinal epithelial cells are the first cell line of interaction with probiotic microorganisms [8]. The activation of these cells leads to the cytokine production, which are responsible for instructing the underlying immune cells, mainly macrophages and dendritic cells. When we analyzed the phagocytic activity of the Peyer´s patches macrophages, less stimulation was shown even when the probiotic bacteria and PFM were administered or for the agonist of TLRs (Figure 1B). This low phagocytic activity may be due to the constant exposition to the normal microbiota, causing that the cells to acquire a tolerization state against microorganisms different to pathogenic bacteria.

Different studies revealed that probiotic lactobacilli could induce upregulation of IL-10 production and cell surface markers of maturation and activation in DCs [21,22]. We analyzed by *in vitro* assays the cytokine production by stimulated macrophages and the importance of the TLR in this production. IL-10 was one of the cytokine studied, considering that this cytokine is an important regulator of the immune system. Distinct mechanisms regulate the expression of this cytokine in the innate and acquired immune systems, NF-κB activation is a major contributor to IL-10 production in macrophages [23]. The probiotic administration induces high levels of IL-10 in peritoneal macrophages, and TLR2 showed be the preferred via for the IL-10 production, however the TLR4 agonist or antagonist did not show any differences (Figure 2A). By previous studies it was demonstrated that the *L. casei* CRL 431 and the PFM were able to activate the NF-kB pathway [24], the results obtained for IL-10 in presence of the TLR agonist reinforce the activation of this pathway.

At the spleen, the macrophages behaviour was different than the peritoneal macrophages. The levels of IL-10 were increased when the cells were stimulated with both probiotics and PFM, but the levels reached by IL-10, were lower than those obtained with peritoneal macrophages. The role of antagonist or agonist for TLR2 or 4 was not evident and maintained elevated the levels of this cytokine (Figure 2C). In Peyer's patches the IL-10 production was more regulated, the probiotic administration did not induce high levels of this cytokine; probably the complex microenvironment in which these cells coexist could explain the different behaviour. The macrophages in this place are in continuous interaction with different bacterial antigens that enter with the diet and with the microbiota. It is known that these intestinal macrophages are major producers of IL-10 and play pivotal roles in the regulation of intestinal homeostasis and inflammation [25]

and the IL-10 production was enhanced by colonization with commensal microbiota [26,27]. It was also suggested that these cells do not express TLR or expressed at very low amount [28] and have a functional blockade in the downstream signalling of TLR [29]. This evidence could explain the low levels of IL-10 obtained for the intestinal macrophages even after probiotic bacteria stimulation, suggesting that these microorganisms do not represent an aggression signal for these cells (Figure 2B).

Other cytokine evaluated was the IL-6 production. This is important in the early resolution phase of innate responses and in the induction of acquired immunity [30,31]. Our results showed that the probiotic or PFM administration induced elevated levels of IL-6 (Figures 2E and 2F), and this level being higher than the control when the TLR2 or TLR4 agonists were added to the spleen and Peyer's patches macrophages culture, suggesting that these receptors are involved in the signals that induce the secretion of this cytokine. In fact, evidence from *in vitro* trials demonstrated that IL-6 production was inhibited when viable or nonviable cultures of *L. casei* CRL 431 interacted with intestinal epithelial cells previously treated with anti-mouse TLR2 antibody [32]. However, the IL-6 production by peritoneal macrophages did not exceed the normal control values (Figure 2D). Probably this last effect is a consequence of the high levels of IL-10 produced by these macrophages, when they were stimulated with the probiotic microorganisms, regulating the IL-6 production.

To evaluate if the activation of the macrophages induced by probiotics mediated through the signals involving TLR2 and TLR4 is also involved in other function of the macrophages, we analyzed by *in vitro* and *in vivo* assays, the antimicrobial capacity of the macrophages.

By *in vitro* assays, we observed that the antimicrobial activation was strain specific. Probiotic bacteria L. casei CRL 431 was able to increase the microbicidal activity of the peritoneal and spleen macrophages and that this increase was TLR-dependent. The administration of PFM or L. paracasei CNCM I-1518, showed a little increase in antimicrobial activity (Figures 3A and 3B). These results could be explained considering that also for the IL-6 production was different according the different strains used. This cytokine are involved in the regulation of the transcriptional factors and is crucial for the macrophages bactericidal and tumoricidal activities [33].

The use of antagonist of TLR2 or 4 had a negative effect on the microbicidal activity. This result is in concordance with the concept that, the TLRs are required for the recognition of the pathogen and the subsequent activation of the innate immune system, but it is important to highlight that the probiotic strains assayed or the PFM administration had an adjuvant effect on the microbicidal activity of macrophages during the infection process even when, the TLRs are blocked.

By *in vivo* studies, we performed an intraperitoneal infection with *Salmonella thyphimurium* and the results confirm the effectiveness of the macrophages, stimulated by the probiotic strains or the PFM, for combating the pathogen, as it was evidenced by a significant diminution in the number of counting of bacterial cells in the spleen and liver during the first 30 hr post-infection (Figures 4A and 4B).

To validate the previous studies, where we demonstrated the implication of the signal induced by probiotic through TLR2 and TLR4 in the phagocytic activity of macrophages, we performed the same assay using TLR2$^{-/-}$, TLR4$^{-/-}$ and MyD88$^{-/-}$ mice. Even though the TLRs are not categorized as phagocytic receptors, our results showed that peritoneal macrophages in TLR2 and TLR4 knockout mice, we showed that the phagocytic activity was significantly diminished in comparison with the wild type mice and that the administration of a probiotic bacteria or PFM was not able to increase the activity of peritoneal macrophages in knockout mice (Figure 5). Furthermore MyD88, turned out to be indispensable for this process.

In addition increased IL-10 production was obtained when peritoneal macrophages were stimulated with a concentration of 10^8 cells/ml of the probiotic *L. casei* CRL 431. Deficiency of TLR4 and 2, produced a significant decrease in the IL-10 production; however the production of this cytokine was almost completely abolished in MyD88-deficient animals (Figure 6). There is a report that shows MyD88 has an important role in the IL-10 induction during Porcine Reproductive And Respiratory Syndrome Virus (PRRSV) infection [34].

These results agree with those obtained in knockout mice, where the adaptor molecule MyD88, demonstrated to have an unquestioned role in the IL-10 production by peritoneal macrophages stimulated with the probiotic bacterium L. casei CRL 431.

These results led us to infer that TLRs play an important role in the phagocytic process and in the cytokine production as IL-10, but they did not play a key effect on the systemic immunity, due to the level of specific anti-OVA IgG did not diminished drastically in TLR knockout mice (Figure 7).

The probiotic strains assayed or the Probiotic Fermented Milk that contain these microorganisms, were able to modulate the macrophages activity through the TLR2 and TLR4 pathway. We demonstrated the influence of these receptors, after oral stimulation with probiotic strains or a PFM in the phagocytic and microbicidal activity of macrophages. We determine that the probiotics through the TLR2 and TLR4 mediate macrophages stimulation to induce an innate immune response, and to send signals to enhance the adaptive immune response. Even when each strain had different behaviour and induces different cytokine profile, the beneficial effect on the macrophages activation was demonstrated.

Acknowledgements

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