Probiotic Intervention Ameliorate Gut Cytokines Profile during Chronic Stress

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Received: January 10, 2018; Published: March 21, 2018

Abstract

Stress results as an essential adaptation necessary for homeostasis, performance and survival, occurring, whenever an individual is faced with an endogenous or exogenous challenge perceived as unpleasant, adverse or threatening. However, when the stimulus is sustained, it can have negative consequences on health with a decreasing in the immune state, predisposing the individual to other diseases. It is known that stress disorders have an important impact on the gut, with cytokine profile altered and the imbalance in the intestinal microbiota. In this sense, previous studies demonstrated that probiotic consumption can improve the gut microenvironment and the immune system in stress condition.

In this work we analyzed the probiotic influence in the improvement of the cytokine release, IL-6, IL-10, IL-1 and TNFα, in the intestinal fluid and others immune cells distant from the gut, as spleen and peritoneal macrophages of stressed mice.

The levels of the cytokine in all compartment analyzed were diminished in stressed mice, showing the immunosuppressed effect induced by the glucocorticoids released during stress. The IL1α was the only cytokine that showed an important increase level in the gut. Is important to highlight the regulatory effect of probiotic, with normalization of the cytokine levels, included the IL1α levels. This last event is relevant considering the key role described for this cytokine in the gut inflammation. The results obtained allow us to suggest the probiotic consumption as a palliative to regulate the immune function during chronic stress.

Keywords: Stress; Probiotics; Cytokine; Gut Immune System; Intestinal Epithelial Cells

Abbreviations

HPA: Hypothalamic-Pituitary-Adrenal Axis; TNFα: Tumor Necrosis Factor alpha; INFγ: Interferon-Gamma; IL: Interleukins; IECs: Intestinal Epithelial Cells; L: Lactobacillus; PBS: Phosphate-Buffered Saline; PFM: Probiotic Fermented Milk; MRS: Mann-Rogosa-Sharp; CFU: Colony Forming Units; BSA: Bovine Serum Albumin; FITC: Fluorescein Isothiocyanate; HBSS: Hank’s Buffered Saline Solution; Gm: Gentamicin; FBS: Fetal Bovine Serum; DTT: DL-Dithiothreitol 1 mmol-1; DMEM: Dulbecco’s Modified Eagle Medium; RPMI: Roswell Park Memorial Institute; PMQ: Peritoneal Macrophages; SMQ: Spleen Macrophages; NKT Cells: Natural Killer Cells

Introduction

Stress is the body's response against stressor agents, which induces a reaction in the brain and physiological responses according to the type of stress (acute or chronic) [1].

Citation: Carolina Maldonado-Galdeano., et al. "Probiotic Intervention Ameliorate Gut Cytokines Profile during Chronic Stress”. EC Gastroenterology and Digestive System 5.4 (2018): 278-290.
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There is no clear consensus about which are the symptoms or biomarkers that define stress, some common signs that are accepted in the scientific literature include clinical, hormonal indicators and other symptoms associated with fatigue, performance decline, insomnia, change in appetite, weight loss and mood disturbances such as irritability, anxiousness, loss of motivation, poor concentration and depression, as well as inflammation and immunosuppression [2].

Chronic stress is associated with negative effects on health and with a decreasing in the immune state, such as reduction in the number of lymphocytes, in the activity of natural killer cells, in the antibody response against virus [3-6]. Other pathologies have been reported in association with chronic stress, among them, cardiovascular disease and autoimmune diseases, diabetes, upper respiratory infections, gastric ulceration, altering intestinal motility and permeability and decreasing in the tissue repair [7-9].

In response to stress, the host released different neurotransmitters, hormones and cytokines that serve as messengers from brain to rest of the body [8].

Studies performed in mice exposure to chronic stress showed changes in both cellular and humoral immunity and alteration in the intestinal microbiota composition [10-12].

Cytokines play an essential role in the cellular activation and communication among the different immune cells. These biochemical mediators are also produced for other non-immune cells, as endothelial, epithelial, and even glial cells [13].

It is known that in stress disorders, like depression, the cytokine profile is altered [14,15]. Increased intestinal permeability, is one of the acute effects induced in untrained adults during exercise, often causing LPS translocation and increases levels of the inflammatory cytokines (TNFα, INFγ) and interleukins (IL1β or IL6), which can eventually result in endotoxemia [16]. Proinflammatory cytokines have a powerful effect by activating the Hypothalamic-Pituitary-Adrenal Axis (HPA) and contribute to the increase in the glucocorticoid levels observed in stress situations [17,18] and in depressive disorders [19,20]. It was also described in mice that many stressors agents cause increases in the circulating cytokines. The social conflict and social disruptions, results in an enhanced immune activity, with increase of IL-6 and antimicrobial activity of splenic macrophages [11,21-23].

Probiotics are defined as live microorganisms which, when administered in adequate amounts confer a health benefit on the host [24]. It was demonstrated that, the regular probiotics consumption, as part of the daily diet, influence the gut mucosa and could be suggested as an alternative to improve the negative impact of stress on the immune system. We demonstrated in healthy mice that probiotic consumption are able to modulate the immune system [25], reinforcing the gut epithelial barrier reducing its permeability and induced an enhance in the local immune response, through the innate immunity with a strong influence on the systemic immunity [26-28]. Probiotic microorganisms can improve the response against enteropathogen infection [29,30], modulate the inflammatory response and influence the composition and activity of intestinal microbiota [31-34].

In previous works, we demonstrated that probiotic lactobacilli consumption improved the intestinal microbiota, the S- IgA levels and restored the number of B and T cells associated to the gut in stressed mice [35].

The aim of the present paper was to evaluate the activation of the immune cells by cytokines determination in a stress model. We analyzed the probiotic influence in the improvement of the cytokine release, IL-6, IL-10, IL-1 and TNFα, in the intestinal fluid and others immune cells distant to the gut of mice subjected to a bi-factorial stress model by movement restriction and food deprivation. It was also analyzed the probiotic impact on the intestinal epithelial cells (IECs) from stressed mice by measurement of IL-6 and IL-10 in the supernatant of IECs culture. The probiotic effect on distant cells from the gut, as spleen and peritoneal macrophages of stressed mice, was also analyzed.
Materials and Methods

Experimental Animals

Five weeks old male BALB/c mice, weighting 25 ± 2g, were obtained from closed bred randomized colony, maintained at CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). Mice were housed in groups of three mice per cage with a controlled atmosphere (22 ± 2°C, 55 ± 2% relative humidity) and a 12h light/dark cycle. Mice were fed with a conventional balanced diet (23% proteins, 6% raw fiber, 10% total minerals, 1.3% Ca, 0.8% P, 12% moisture and vitamins) and water ad libitum until the experimental procedure was initiated.

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 CERELA, protocol number: CRL-BIOT-Li-2011/1A, and all experiments comply with the current laws of Argentina.

Stress protocols and experimental groups

The stress was induced during eleven consecutive days by two different and simultaneous factors: immobilization and food deprivation, as was described in previous paper [35]. Briefly mice were placing inside cylindrical plexiglass containers (10 cm length, 3.5 cm (internal diameter)) with ventilation holes to prevent hyperthermia, being the time of the restriction of 3h, from 11:00 to 14:00h. During this time, mice were allowed to move only back and front in the tube but could not turn around. Food restriction was it carried out during the period of higher activity of the mouse corresponding to 20:00 - 8:00 h (12hs). During this time, only water was accessible to the mice. The experimental groups were formed as described below: 1) Normal Control group (NC): The animals received balanced diet and water ad libitum. 2) Stressed group (S). 3) Stressed group plus probiotic Lactobacillus casei CRL 431 (S + CRL431). 4) Stressed group plus probiotic Lactobacillus paracasei CNCM I-1518 (S + CNCM I-1518). 5) Stressed group plus probiotic fermented milk (S + PFM).

Each experimental group consisted of three animals. At day 12 of the experiment, mice from each group were sacrificed by cervical dislocation and serum, intestinal fluid from the small intestine and small intestine tissue were taken. Serum and intestinal fluids were stored at -18ºC until used. Samples taken for the histological studies were processed immediately. The increases of corticosterone levels in serum were used to evaluate the stress protocol.

Probiotics lactobacilli assayed

The probiotic microorganisms assayed were: Lactobacillus (L) casei CRL 431 isolated from infant feces, deposited in the American Type Culture Collection (ATCC), number 55 544. The probiotic strain was maintained and controlled at CERELA culture collection. Overnight cultures were grown in sterile Mann-Rogosa-Sharp (MRS, Britannia, Buenos Aires, Argentina) broth at 37ºC. After incubation, cells were harvested by centrifugation at 5,000 g for 10 minutes, washed three times with fresh sterile phosphate-buffered saline (PBS) 0.01M, and resuspended in sterile 10% (v/v) non-fat milk. L. casei CRL 431 was administered to mice in the drinking water at a concentration of 1 X 10^8 colony forming units (CFU)/ml during the experiment, according to standard protocols used in the laboratory [36]. The bacterial suspension was prepared and administered daily at 9:00h. To ensure viability and the number of bacterium administered, the CFU were counted daily. The other probiotic strain assayed was Lactobacillus paracasei CNCM I-1518. It was ceded and deposited by DANONE SA Argentina in the CERELA culture collection and was administered to mice in the same condition described for the L. casei CRL 431 strain. We also analyzed a commercial probiotic fermented milk (PFM) containing yoghurt starter cultures (Lactobacillus delbrueckii subsp. bulgaricus 108 colony- forming units (CFU)/ml, Streptococcus thermophilus 108 CFU/ml) and the probiotic bacterium Lactobacillus paracasei CNCM I-1518 (10^6 CFU/ml). It was administered ad libitum during the experimental time.

Histological samples

At the end of each administration period both, test and control animals were sacrificed. The small intestine was removed and processed according to Sainte-Marie’s technique for paraffin embedding [37].
Cytokine-positive cells detection in lamina propria of the small intestine

IL-1α, IL-10, IL-6 and TNFα producing cells were studied in all the groups by an indirect immunofluorescence assay. After deparaffinization and rehydration, paraffin sections (4 μm) were incubated with a 1% blocking solution of bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) for 30 minutes at room temperature. They were washed three times in PBS and incubated with normal goat serum (dilution 1:100) for 30 minutes. Rabbit anti-mouse for each cytokine assayed were applied to the sections for 60 min at room temperature. Then the slices were washed twice in PBS and incubated for 45 minutes with a 1:100 dilution of the goat anti-rabbit antibody conjugated with Fluorescein Isothiocyanate (FITC) (Jackson Immuno Research Labs Inc.) at room temperature and washed twice in PBS. The number of fluorescent cells was counted in 30 fields at 1000X magnification and results were expressed as the number of positive fluorescent cells per ten fields.

Determination of the cytokines levels in the small intestinal fluids

Intestinal fluids were collected from the small intestines by washing with 1 ml of PBS and immediately centrifuged at 5000g for 15 minutes at 4°C. The supernatant was separated and stored at -20°C until determination of cytokine (IL-10, IL-6) concentrations using BD OptEIA cytokine ELISA set (BD Bioscience, San Diego, USA). The results were expressed as the concentration of each cytokine in the intestinal fluid (pg/ml).

Intestinal epithelial cells isolation: ex vivo assays

To analyze the impact of the probiotic bacteria assayed on the intestinal epithelial cells (IECs), the IL-6 and L-10 release were measured. The IECs isolation was performed, at day 12 of the experiment, where the animals from the different experimental groups of mice were sacrificed and the IECs were isolated from the small intestine. The small intestine was removed and then was washed with cold PBS + gentamicin (Gm, 100 µg/ml) to remove the intestinal content. Peyer’s patches were discarded and the rest of the intestine was opened longitudinally to expose the mucosal surface. The samples were washed five times with ice-cold Hank’s buffered saline solution (HBSS) + Gm at 4°C. Each sample was cut into small segments and placed in ice cold HBSS + fetal bovine serum (FBS) 8% + Gm. After that, the samples were treated with DL-dithiothreitol 1 mmol−1 (DTT, Biochemika, Fluka) + EDTA (Sigma, St. Louise, USA) 10 mmol-1 in the same solution composed by HBSS + FBS 8% + Gm 100 μg ml−1, and they were incubated 15 min at 4°C. Supernatants were discarded and the pellets were incubated in erlenmeyers with 15 ml of HBSS + FBS 8% + Gm 100 µg ml−1 + EDTA 30 mM, using a magnetic bar to shake the samples during 15 minutes. Supernatants were separated and left to settle for two minutes; to allow the sedimentation of non-digested large fragments. The clarified supernatants containing IECs were collected and centrifuged at 300g during 5 minutes. The pellets were washed twice, and resuspended with Dulbecco’s Modified Eagle Medium (DMEM High Glucose 1X, Gibco-Invitrogen). Trypan blue (0.4%) exclusion was used to assess cell viability. Purity of isolated IECs was evaluated by incubation of cell suspensions with phycoerythrin-conjugated anti-mouse CD45 (BD Biosciences Pharmingen, USA). 20,000 to 30,000 events were analyzed by flow cytometry with a BD FACSCalibur™ flow cytometer and the percentage of positive contaminating hematopoietic (CD45+) cells observed was 19.22%.

These cell suspensions were transferred to 96-well cell culture plates and incubated for 8 h (37°C, 5% CO₂). Supernatants were recovered and conserved at -70°C until cytokine (IL-6 and IL-10) determination by ELISA test performed in the same way described for cytokines from intestinal fluid.

Cytokine determination in Peritoneal and Spleen Macrophages culture

Macrophages from the peritoneal cavity were harvested by vigorous lavage with 5 ml of cold PBS 0.01M. Then the suspension was centrifuged and washed twice with PBS and the concentration was adjusted at 1 x 10⁶ cells/ml in Roswell Park Memorial Institute 1640 (RPMI) medium.
Spleen from each group were collected in 5 ml of HBSS solution containing FBS and aseptically disrupted \([25,28]\). The cells were harvested by centrifugation at 800 - 1000g for 15 minutes at 4\(^\circ\)C and red blood cell were lysed using lysing buffer (Sigma, St Louis, USA). Peritoneal and spleen macrophages from different groups of mice were separated from the mononuclear population using their adherence property in TC-Plates (6 wells, sterile with LID Cellstar Greiner bio-one) (1h; 37\(^\circ\)C; 5% CO2). The adherent cells (macrophages and DC) were recovered by scraping and the final concentration was adjusted at 1 x 10^6 cells/ml/wells in RPMI medium. Peritoneal and spleen macrophages were incubated (18h; 37\(^\circ\)C; 5% CO2). 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).

**Statistical analysis**

Statistical analyses were performed for 3 animals of each group. The experiment was repeated three time \(n = 9\) using ANOVA GLM followed by a Tukey’s posthoc test using MINTAB 14 software (Minitab, Inc., State College, PA, USA), \(p < 0.05\) was considered significant. Values are the means of 3 independent trials (no significant differences were observed between individual replicates) ± standard deviation (SD) from \(n = 9\). Each immunohistochemical determination for each mouse, the results were obtained from two blind counts by two different researchers.

**Results**

**Cytokines producing cells in lamina propria of the small intestine**

We observed that stressed mice showed at the end of the experimental time (12 days), increases in the number of IL-1\(\alpha\)+ cells in lamina propria (Figure 1A). Others cytokine producing cells determined (IL-6+, IL-10+ and TNF- \(\alpha\)+ cells) were diminished in stressed mice compared with non-stressed mice group (Figure 1 B, C and D respectively). The consumption of any of the two probiotic strains or the PFM during stress normalized the values of cytokines producing cells in relation with the non-stressed control. PFM induced the major number of TNF\(\alpha\)+ cells in lamina propria of the small intestine, being even higher levels than the control group (Figure 1).

![Figure 1](image)

**Figure 1**: Probiotic effect on cytokine producing cells in the small intestine. The numbers of cytokine producing cells were determined by indirect immune fluorescence on the small intestine tissue slides of mice from different groups: Normal control (NC), Stressed (S), stressed plus L. casei CRL 431 (S+CRL 431), stressed plus L. paracasei CNCM I-1518 (S + CNCM I-1518) or stressed with PFM (S+FM). The results were expressed as the number of A) IL-1 positive cells, B)IL-6 positive cells, C) IL-10 positive cells and D) TNF\(\alpha\) positive cells, per 10 fields of vision (1000X). Data correspond to the means ± SD of 9 animals from three separated experiments. * and # represent significant differences\((p< 0.05)\) with NC and OC, respectively.
IL-6, IL-10 levels in the intestinal fluid

We analyzed the release of IL-6 and IL-10 in the intestinal fluid. In mice from the stressed control group IL-6 was increased in the intestinal fluid and the IL-10 was diminished compared with the non-stressed control group. The consumption of the two probiotic strains maintained similar levels of IL-6 and IL-10 compared with the non-stressed mice. The PFM induced highest IL-10 levels (Figure 2).

![Figure 2: IL-6 and IL-10 determination in the intestinal fluid. At day 12 of the experiment, intestinal fluids were collected from the small intestines of NC and different groups of stressed mice (S, S+ CRL 431, S+ CNCM I-1518 or S+ PFM). IL-6 and IL-10 concentrations were determined in the intestinal fluid using BD OptEIA cytokine ELISA set. Results were expressed as pg/ml of each cytokine. Data correspond to the mean± SD of 9 animals from three separated experiments. * and # represent significant differences(P < 0.05) with NC and S groups, respectively.]

Probiotic effect on IL-6 and IL-10 levels from IEC’s, peritoneal and spleen macrophages in the stressed control group

The levels of IL-6 produced by IECs increased significantly in stressed mice compared with the non-stressed mice. The probiotic or PFM consumption was able to maintain IL-6 levels to similar values to the non-stressed control group. The IL-10 levels were also affected by stress conditions, which led to a significant increase in this cytokine compared to the control group. L. casei CRL431, L. paracasei CNCMI-1518 and PFM consumption resulted in a reduced IL-10 production with values even below the non-stressed control (Figure 3A).

Levels of IL-6 in the PMQ supernatant decreased in all animals that were subjected to bi-factorial stress protocol. Groups of mice given with probiotics or PFM, evidenced a IL-6 increases, being the L. paracasei CNCMI-1518, the only probiotic strain that reached to the levels of the control group. IL-10 was increased in the PMQ supernatant culture of all groups subjected to bi-factorial stress protocol in relation with the control group. Among the groups receiving probiotic no significant differences were found compared with the stressed groups of mice (Figure 3B).

The spleen macrophages showed a similar behavior those peritoneal macrophages referred to IL-6 levels. In this case the both probiotic strain were able to restore the normal IL-6 levels (Figure 3C).

The IL-10 levels were not modified with stress, however the PFM and the L. paracasei CNCMI-1518 consumption increases the IL-10 values significantly with respect to the others group (stressed and non-stressed mice) (Figure 3D).

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Discussion

The damage caused by stress on the body is directly related to the duration of this state. The mechanisms activating in acute or chronic stress are different. In the chronic stress it is well described changes in the mechanisms regulating the immune response, variations in the body weight, adrenal glands hypertrophy, diminution in the thymus, and spleen weight and in lymph nodes [38-43]. The immune response in the stress is associated with an immunosuppression; such as reduction in lymphocyte proliferation, in the activity of NKT cells (Natural Killer Cells) [44-47]. Lymphocytes, monocytes or macrophages and granulocytes, expressed glucocorticoids receptors that bind cortisol interfering in the NF-kB function, which regulates the activity of cytokine-producing immune cells. The activation of adrenergic receptors induces an altered profile of proinflammatory and regulators cytokines, indicating that stress induces changes in the cytokine genes expression leading an immune dysregulation [48,49].

Acute stress inhibits Th1 immune response, mainly by the inhibition of IL-12. Furthermore in chronic stress, there is an unbalance in the population to which changes to Th2 response. Glucocorticoids released during stress has also been shown to diminish the TLR expression, leading to immune system depression, evidenced by decreased proliferation, migration and T cell toxicity as well as cytokine production and reduction the capacity host antimiobacterial defense [50,51].

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In previous work, we showed that in our chronic stress model, immune parameters were affected, such as the number of IgA+, CD4+ and CD11b+ cells and in the S-IgA levels of the small intestine. Probiotic consumption in stressed mice was able to improve intestinal microenvironment, inducing a positive balance in the intestinal microbiota [35].

In a present paper we explored the changes on the cytokine profile during stress and whether or not the probiotic consumption influencing this profile.

We analyzed the effect on the cytokines at the intestinal epithelial and immune cells associated to the small intestine. The epithelial cells of the gut and leukocytes have a strong communication and response to different signals that induce cytokine release that influence cell migration, differentiation, replication, or activation of cell-intrinsic defenses. The immunosuppressive effect of glucocorticoids induced by stress is described in the literature, by inhibition mainly of the inflammatory response, as well as, the increased antibody production [52,53], being a risk factor for infection or other pathologies that can occurs in chronic stress.

At the intestinal level cytokine response is different among different cells that inhabit the intestine, due to the complex network of signals allowing cytokines to influence the behavior of the immune cells.

Changes in the equilibrated production of cytokine or the disruption of one particular cytokine in these networks may differ depending on the cell types and the intestinal regions involving the environmental microbiota [54].

Intestinal epithelial cells are very sensitive to hormonal changes induced during stress, responding to this stimulus with increased IL-6 and IL-10 production.

The immune cells from the lamina propria of the small intestine showed different behaviour than IECs. The IL-1α was the cytokine that showed an important increment, while the IL-10, TNFα and IL-6, decreased in stressed host.

The IL-1α from macrophages and DCs can induce the recruitment of neutrophils while in IECs is released during necrosis [55,56]. The increases of IL-1α are related with the exacerbation of intestinal bowel disease (IBD). Recent reports showed that IL-1α deficient mice developed a less severe disease; this result suggests the important relation between high IL-1α levels with the intestinal inflammation [57].

It was demonstrated that the injection of LPS increased the IL-1α expression in brain of stressed animals, concluding the important role in the communication between immune system and central nervous system through the biological messenger as are the cytokine [58].

We demonstrated that the probiotic consumption had a regulatory effect on the IL-1α cytokine levels accompanied with normal levels of IL-10, IL-6 and TNFα in the stress model assayed, these effects helps to restore intestinal homeostasis, allowing the IL-1α levels restoration at the intestine that suggest an important regulation on the other cytokines.

Recently studies performed in patients with ulcerative colitis suggested an important correlation between IL-10 levels and the response to treatment with glucocorticoids (GC), being the low IL-10 levels associated with a poor response due to GC [59]. As it well known IL-10 plays a preponderant role as immune regulatory cytokine; thus the deficiency of this cytokine increases intestinal permeability [60]. These data suggest that one of the mechanisms by which IL-10 exerts its anti-inflammatory effect could be mediated by regulating the barrier function at the intestinal level. However, the molecular mechanisms of these actions, and how the glucocorticoids affect this response, are still not well understood.

The IL-6 cytokine is induced and released by IECs, it is from increased from crevicular exudate (the plasma fluid between the gums and the teeth) after accumulation of oral bacteria and also in psychological depression chronic stress [61-63]. These studies concluding that stress play an important role in the immune system communication with the central nervous system [58]. We found that probiotics consumption during stress protocol reduced the number of IL-1α in lamina propria, being the effect more evident for the supplementation with the probiotic strain S+CNCM I-1518 and with PFM, suggesting an improvement in the lamina propria of the small intestine that would reinforce the intestinal barrier by diminution of the inflammatory response.

Further studies reports that depression and certain related stress situations diseases or other negative emotions stimulate the production of proinflammatory IL-6 activity [64-67]. The IL-6 release is also associated with a more severity of upper respiratory symptoms among persons infected with influenza A virus [68]. This cytokine has a positive effect on the glucocorticoids levels during chronic stress; however the behavior of this cytokine at the intestinal level is more complex and still understood. IL-6 plays an important role in the switch of IgM+ B lymphocytes to IgA+ B lymphocytes. This fact is relevant in the gut, because the IgA is the most important immunoglobulin at the mucosal surface and it has a regulatory function at the intestinal barrier. In a previous report [35], we demonstrated that the production of IgA was diminished in stressed mice and the present results showing an increase for IL-6 reinforce the fact that probiotics are able to restored the IL-6 production accompanied with the increases in the IgA level at the intestinal mucosal, suggesting that the regulatory changes in the cytokine profile at this level would be different than other sites such as brain or in serum.

Considering these results we are conclude that the consumption of probiotics, including in a variety of functional food, are an excellent option to ameliorate the deregulation in the cytokines expression at the intestine during chronic stress.

Acknowledgements

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Argentina (Dr. Carolina Maldonado (PIP 806). Agencia Nacional de Promoción Científica y Tecnológica (PICT 2964). Secretaria de Ciencia, Arte e Innovación Tecnológica, Universidad Nacional de Tucumán, Argentina (PIUNT 26D/529). The authors thank Danone Argentina to provide the strains Lactobacillus paracasei CNCM I-1518.

Conflict of Interest

The authors have no financial conflicts of interest.

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