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Influence of a probiotic lactobacillus strain on the intestinal ecosystem in a stress model mouse

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ABSTRACT

Daily exposure to stressful situations affects the health of humans and animals. It has been shown that psychological stress affects the immune system and can exacerbate diseases. Probiotics can act as biological immunomodulators in healthy people, increasing both intestinal and systemic immune responses. The use of probiotics in stress situations may aid in reinforcing the immune system.

The aim of this study was to evaluate the effect of a probiotic bacterium on the gut immune system of mice that were exposed to an experimental model of stress induced by food and mobility restriction.

The current study focused on immune cells associated with the lamina propria of the intestine, including CD4+ and CD8+ T lymphocytes, CD11b+ macrophages, CD11c+ dendritic cells, and IgA+ B lymphocytes, as well as the concentrations of secretory IgA (S-IgA) and cytokine interferon gamma (INF-γ in intestinal fluid. We also evaluated the probiotic's influence on the gut microbiota.

Probiotic administration increased IgA producing cells, CD4+ cells in the lamina propria of the small intestine, and S-IgA in the lumen; it also reduced the levels of IFN-γ that had increased during stress and improved the intestinal microbiota as measured by an increase in the lactobacilli population.

The results obtained from administration of the probiotic to stressed mice suggest that the use of food containing these microorganisms may work as a palliative to reinforce the immune system.

1. Introduction

Stress can be defined as the physiological reaction of an organism to external or internal stimuli in which the body initiates various defense mechanisms to maintain homeostasis (Ramsey, 1982; Cruz et al., 2012).

Daily stress situations affect the normal health of humans or animals by exacerbating or promoting the development of disease, thus increasing the risk of cancer, autoimmune diseases or infections (Bartrop et al., 1977; Mason, 1991; Maunder, 2005 and Cohen et al., 2012).

The damage induced by stress varies depending on the time at which the body is exposed to the stressor agent (Dhabhar, 2003).

Studies performed in experimental animal models have demonstrated that repeated exposure to a stress situation induces changes in cellular and humoral immunity (Bauer et al., 2001; Du et al., 2010) and also affects intestinal microbiota (Bailey et al., 2011; Sudo et al., 2004).

In stress situations, the major hormones released are glucocorticoids and catecholamines. These hormones alter immune functions such as antigen presentation, leukocyte trafficking and proliferation, antibody secretion and cytokine release (Dhabhar et al., 1995). The hormones produced at high levels during stress, such as cortisol, influence a range of factors including anti-inflammatory responses, the metabolism of carbohydrates, fats, and proteins, and gluconeogenesis. Similarly, catecholamines work in concert with the autonomic nervous system to exert regulatory effects on cardiovascular, pulmonary, hepatic, and skeletal muscles.

As a consequence of long-term exposure to glucocorticoid, glucocorticoid receptor resistance in hosts leads to a decrease in the sensitivity of immune cells and impairs downregulation of the inflammatory response (Miller et al., 2002).

The immune system is significantly influenced by stress; therefore, increasing the immune system potential could be beneficial to the host's health. In this sense, probiotic microorganisms represent an option to enhance immunity in a stress situation.

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2001). They can modulate the immune system in healthy people by increasing the mucosal and systemic immune responses (Galdeano and Perdigón, 2004), reinforcing the epithelial barrier by reducing its permeability, and enhancing the local

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immune response, mainly through innate immunity, although systemic immunity can be affected as well (Galdeano and Perdigon, 2006, Galdeano et al., 2007; Maldonado Galdeano et al., 2011). Probiotic microorganisms can improve the immune response against infection (Castillo et al., 2011; Maragkoudakis et al., 2010), modulate the inflammatory response, and influence the composition and activity of intestinal microbiota (Chaves et al., 2011, de Moreno de LeBlanc et al., 2008).

The aim of this paper was to evaluate the effect of a probiotic bacterial strain, *Lactobacillus casei* CRL 431, when orally administered to BALB/c mice in an experimental model of stress induced by food and mobility restriction. We analyzed the changes in the intestinal microenvironment induced by stress and whether the probiotic strain improved the gut mucosal immunity. We focused our study on immune cells associated with the lamina propria of the small intestine such as CD4 and CD8 T lymphocytes, CD11b+ macrophages, CD11c+ dendritic cells, and IgA+ B lymphocytes, as well as the levels of secretory IgA (S-IgA) and cytokine interferon gamma (IFN-γ) in the intestinal fluid. We also evaluated the probiotic influence on the microbiota.

2. Methods

2.1. Experimental animals

Male BALB/c mice were obtained from the closed random bred colony at CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). Mice of 5 weeks of age were housed in groups of three mice per cage. All animals were maintained in a room with a regular 12-h light/dark cycle at 20 ± 2 °C over the course of the experiment (11 days). The animals received 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.

2.2. Stress protocols and experimental groups

We believe that stress is generally a result of more than one factor acting simultaneously. In this work, stress was induced by two different and simultaneous factors: mice were stressed via immobilization by placing them inside cylindrical plexiglass containers (10 cm length × 3.5 cm (internal diameter)) with ventilation holes to prevent hyperthermia. The animals were allowed to move back and forth in the tube but could not turn around. The duration of the restraint cycle was 3 h, from 11:00 to 14:00 h. The other stressor agent employed was food restriction for 12 h (20:00–8:00 h), a period corresponding to the active phase of mice (night) when they usually receive food. During the food restriction time, only water was accessible to the mice. Both stress protocols were carried out over eleven consecutive days.

There were four experimental mice groups. Normal Control group (NC): The animals received balanced diet and water *ad libitum*. During the restrained time, mice in this group were left undisturbed in their home cages. Stressed group (S): Animals were subjected to the stress protocol detailed previously. Stressed group plus probiotic (S + P): Stressed mice that received a suspension of probiotic bacterium *Lactobacillus casei* CRL 431 in drinking water over the course of the experimental period. Non-stressed group plus probiotic (NC + P): Non stressed mice received a suspension of probiotic bacterium *Lactobacillus casei* CRL 431 in drinking water over the course of the experimental period.

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 large and small intestine samples were taken. Serum and intestinal fluids were stored at −18 °C until used. Samples taken for the histological studies and microbiota analysis were processed immediately.

2.3. Probiotics administration protocol

Lactobacillus (L) casei CRL 431 was isolated from infant feces, identified by rapid fermentation test API 50 CH Carbohydrates (BioMerieux, France) and molecular biology as *L. paracasei* subsp. *paracasei*, and deposited with 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, Britania, Buenos Aires, Argentina) broth at 37 °C. After incubation, cells were harvested by centrifugation at 5000*g* for 10 min, washed three times with fresh sterile phosphate-buffered saline (PBS) 0.01 M, 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 1×10^8 colony forming units (CFU)/ml during the experiment, according to standard protocols used in the laboratory (Perdigón et al., 2002). The bacterial suspension was prepared daily at 9:00 h to ensure viability and strictly maintain the number of CFU administered every morning.

2.4. Levels of corticosterone in serum

Serum samples for corticosterone hormone determination were collected at the same time for each experiment and were evaluated in all experimental groups simultaneously. Blood samples were obtained by cardiac puncture within 1–2 min after euthanasia and immediately centrifuged for 25 min at 5000 g. The serum samples were stored at −18 °C until further use. A normal control was included to allow for the possibility that cervical dislocation could cause changes in corticosterone levels.

Serum corticosterone levels were measured by radioimmunoassay as described previously (Armario and Castellanos, 1984), with one modification: corticosterone-binding-globulin was denatured by heating the samples at 70 °C for 30 min. Inter- and intra-assay coefficients of variation were 14% and 11%, respectively.

2.5. Histological samples

The small intestine samples were fixed in PBS-formaldehyde solution 10%, pH 7. After fixation, the tissues were dehydrated and embedded in paraffin using conventional methods (Sainte-Marie, 1962). Serial paraffin sections (4 μm) were made and used for hematoxylin-eosin staining to analyze by optical microscopy at 100X magnification.

2.6. Direct immunofluorescence for IgA+ cells, CD4+ and CD8+ T lymphocytes, CD11b+ macrophages and CD11c+ dendritic cells in lamina propria of small intestine

The number of IgA+ B lymphocytes, CD4+ and CD8+ T lymphocytes, CD11b+ macrophages and CD11c+ dendritic cells were determined by direct immunofluorescence assays. The markers used are specific to identify each cell type. After deparaffinization using xylene and rehydration in a decreasing ethanol gradient, small intestine slices from different experimental groups were incubated with anti-mouse IgA-α–chain monospecific antibody conjugated

with fluorescein isothiocyanate (FITC, Sigma, St. Louis, USA) for IgA+ cells, monoclonal antibodies conjugated with FITC (Cedarlane, Ottawa, Canada) for CD4+ or CD8+ T lymphocytes (clone CT-CD4 or H57-597, respectively), or FITC monoclonal antibodies (BD Pharmingen, California USA) for CD11b+ or CD11c+(clone M1/70 or HL3, respectively). The results of 3 mice per group from three independent trials were expressed as the number of positive cells per 10 fields of vision (magnification 1000×) using a fluorescent light microscope.

2.7. ELISA assays for secretory IgA (S-IgA) and interferon gamma (IFN-γ) levels in small intestinal fluids

Intestinal fluids were collected according to standard protocol (Castillo et al., 2012) from the small intestines of animals from each experimental group with 1 ml of 0.85% of NaCl and were centrifuged immediately at 5000 g for 15 min at 4 °C. The supernatant was recovered and stored at −18 °C until use for S-IgA and IFN-γ measurements. ELISA tests were performed in triplicate for each animal from each experimental group.

To measure the concentration of total S-IgA, an ELISA test was performed. Ninety-six well microplates were coated with a goat anti-mouse IgA affinity-purified antibody (BETHYL Laboratories INC., Montgomery, Tx, USA) by incubation at 37 °C for 1 h with carbonate-bicarbonate buffer (pH 9.6). The plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 0.5% non-fat dry milk in PBS for 1 h at 25 °C. After blocking, plates were washed and incubated with either 50 µl of standard kappa IgA (Sigma, St. Louis, USA) or 50 µl of intestinal fluid sample for 2 h at 37 °C. Plates were washed and incubated in the presence of horseradish peroxidase-conjugated anti-mouse-IgA (α chain specific antibodies, Sigma, St. Louis, USA, product N° 4789) for 1 h at 37 °C. Plates were washed again and developed using trimethylbenzidine (TMB) reagent containing peroxide (BD Biosciences, San Diego, USA). Reactions were stopped with H_2SO_4 (2 N). The absorbance was read at 450 nm using a VERSA Max Microplate Reader (Molecular Devices, USA).

The levels of IFN-γ were measured using BD OptEIA TM (Product N°555138) mouse IFN-γ ELISA kits (BD Biosciences, San Diego, USA) following the manufacturer's instructions. The results were expressed as the concentration of IFN-γ (pg/ml) or S-IgA (µg/ml) in the intestinal fluid.

2.8. Analysis of the intestinal microbiota

Large intestines from mice in control and test groups were aseptically removed, weighed and placed in sterile tubes containing 5 ml of peptone water (0.1%). The samples were homogenized immediately under sterile conditions. Serial dilutions of the homogenized samples were performed and aliquots (0.1 ml) of dilutions were spread onto the surface of the following agarized media: Reinforced Clostridial Agar (RCA, Britania, Buenos Aires, Argentina) for total anaerobic bacteria, Mann-Rogosa-Sharp (MRS Britania, Buenos Aires, Argentina) for total lactobacilli (Britania, Buenos Aires, Argentina), and MacConkey for total enterobacteria. MacConkey and MRS agar were aerobically incubated at 37 °C for 24 h and 48 h, respectively. The other culture media were anaerobically incubated at 37 °C for 72–96 h.

2.9. Statistical analysis

Statistical analyses were performed by ANOVA GLM followed by a Tukey's posthoc test using MINITAB 14 software (Minitab, Inc., State College, PA, USA), and $p < 0.05$ was considered significant. Unless otherwise indicated, all values are presented as the means of 3 independent trials (no significant differences were observed between individual replicates) ± standard deviation (SD) with $n = 9$. For each immunohistochemical determination and for each mouse, results were obtained from two individual blind counts (by two different researchers).

3. Results

3.1. Corticosterone levels

Analysis of corticosterone in serum showed significant increase in hormone level in the stressed group of mice compared to that in the control group. This result validates our stress protocol. However, probiotic administration in the S + P group did not affect levels of this hormone in the serum (Fig. 1). In addition, administration of the probiotic bacterium in non-stressed animals revealed similar corticosterone values to those in the normal control group (data not shown).

3.2. Histology of the small intestine

Analysis of the histology samples from different experimental groups showed changes in the intestinal villi of stressed mice, which had shorter villi length in comparison to those of the normal control mice. This effect was reversed with probiotic administration. We did not observe edema or dysplasia of epithelial cells in any of the groups in our study. We observed a decrease in leukocyte infiltration in the stress groups compared to that in the NC and NC + P groups. Leukocyte infiltration was restored in the stress group after probiotic administration (Fig. 2).

3.3. Determination of IgA+, CD11b+ and CD11c+ cells and CD4+ and CD8+ lymphocytes in the lamina propria of the small intestine

Analysis of IgA+ B lymphocyte expression revealed that the number of IgA+ cells changed significantly under stress conditions. There was a significant decrease in the number of IgA+ cells in the lamina propria of the small intestine in the S group compared to the number in the NC group. *L. casei* administration in stressed mice (S + P) restored IgA + cell numbers to near normal levels. Normal mice given the probiotic showed a significant increase in the number of IgA+ cells compared to cell numbers in other groups (Fig. 3A and Fig. 3Ba–d).

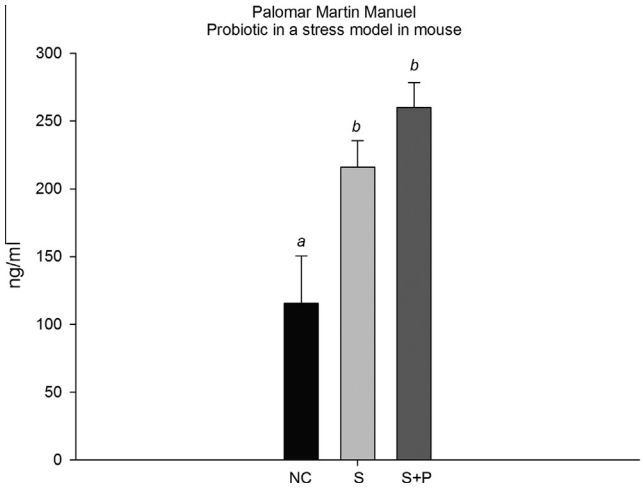

Fig. 1. Serum samples from all experimental groups were collected within 1–2 min after cervical dislocation and stored at −18 °C until use. The stress protocol induced a significant increase in corticosterone levels in the S and S + P groups when compared to levels in the NC group. (a and b) Means for each group without a common letter differ significantly ($P < 0.05$). NC (normal control), S (stress group), S + P (stress + probiotic).

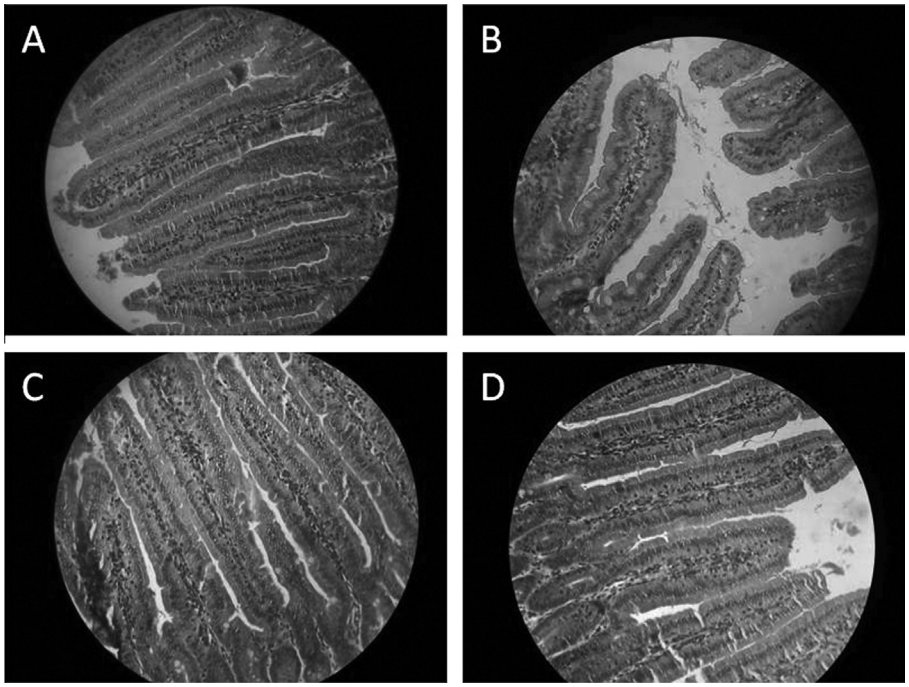

Fig. 2. Histological study of the different experimental groups. Histological slices from small intestines of mice were examined after staining with hematoxylin-eosin. Each microphotograph was taken at 100× magnification and corresponds to one mouse that represents the majority (more than 75%) of images obtained from individual animals within each group. (A) Normal control group (NC), (B) Stressed control group (S); the intestinal villi are short and leukocyte infiltration is lower than in the NC group (C) Normal control group given *L. casei* CRL 431 (NC + P); the villi length are similar to the NC group and (D) Stressed mice given probiotic bacterium (S + P); probiotic administration improved the histological alterations caused by stress and showed histological characteristics similar to those in the NC groups.

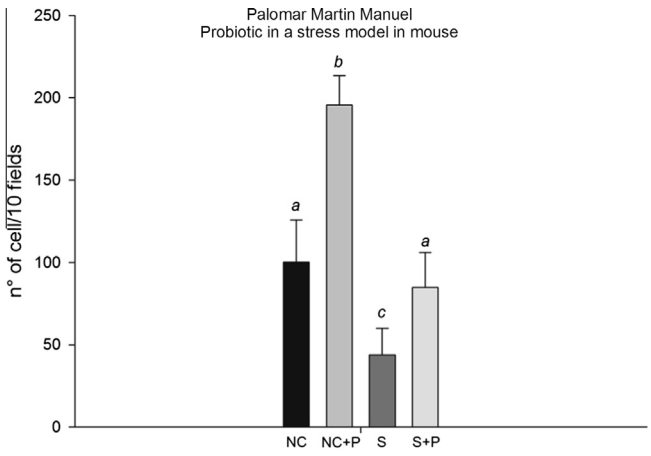

Fig. 3A. Effect of the stress protocol on IgA+ cell number in the lamina propria of the small intestine. IgA+ cell numbers were determined by direct immunofluorescence on small intestine tissue samples from mice from different experimental groups: Normal control (NC), normal control given *L. casei* (NC + P), stressed control (S) and stressed mice given *L. casei* (S + P), Results were expressed as number of positive cells counted in 10 fields of vision at 1000X magnification. Values are means for $n = 9 \pm$ SD mice from each group. (a–c) Means for each value without a common letter differ significantly ($P < 0.05$). The number of IgA+ cells decreased significantly in stressed animals (S) in respect to that in NC animals ($P < 0.05$), while the stressed animals fed the probiotic (S + P) showed an increase in the number of cells ($p \leqslant 0.05$).

After determining CD4+ and CD8+ T lymphocyte numbers, we found that the number of CD4+ cells was significantly diminished in the stress group compared to the control group ($P < 0.05$). Oral administration of the probiotic increased the number of these cells in the S + P group compared to that in the S group, but showed no difference from the NC group cell numbers ($p \leqslant 0.05$) (Table 1).

The number of CD8+ T lymphocytes is significantly reduced in the stress protocol group compared to the NC group. There were no differences when comparing other groups (NC vs. NC + P vs. S + P) ($p \leqslant 0, 05$) (Table 1).

We analyzed the expression of CD11b+ and CD11c+ markers to determine macrophage and dendritic cell numbers, respectively. The number of CD11b+ cells decreased significantly in stressed mice compared to the control mice. Stressed animals that received the probiotic bacterium had a significant increase in the number of these cells compared to the normal control ($p \leqslant 0.05$) (Table 1). With respect to CD11c+ cells, we found that the stress protocol produced a significant increase in these cells compared to the NC group. Probiotic administration maintained the number of these cells and revealed values similar to those in the normal control group (Table 1).

3.4. Determination of S-IgA and IFN-γ levels in small intestinal fluids

Analysis of S-IgA levels in the intestinal fluid showed that levels of this immunoglobulin decreased significantly in animals subjected to stress conditions (9.73 ± 1.4 ug/ml) compared with the normal control group levels (40 ± 7 ug/ml).

The probiotic administration in stressed mice increased the levels of S-IgA (20.4 ± 1.6 ug/ml), although these values were still lower than those obtained for the control group. The NC + P experimental group showed values significantly higher than those for the NC group (Fig. 4).

The IFN-γ analysis showed that levels of this cytokine in the intestinal fluid were significantly increased in NC + P mice in comparison to the normal control group levels. There was no difference in IFN-γ levels between the two stress groups, but both groups showed increased IFN-γ levels compared to levels in the NC group (Fig. 5).

3.5. Microbiota analysis

Examination of the intestinal microbiota showed that probiotic administration increased the number of total anaerobes in NC + P, while the other three groups showed no changes.

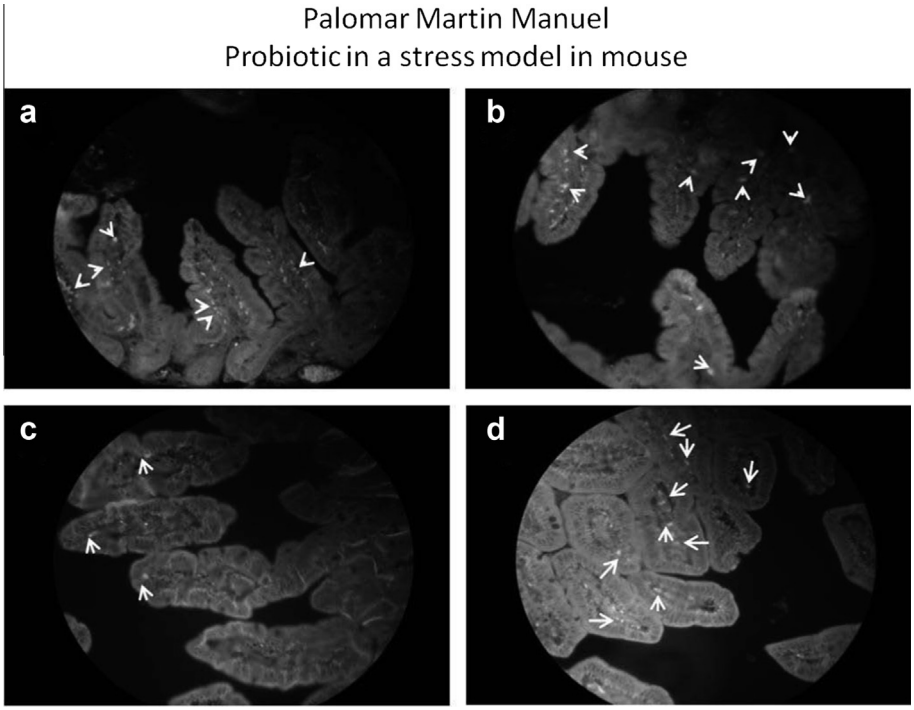

Fig. 3B. Each microphotograph was obtained using 100× magnification and corresponds to one mouse that represents the majority (more than 75%) of images obtained from individual animals in each group. (a) The Normal control group (NC), (b) normal control given *L. casei* (NC + P), (c) stressed control (S) and (d) stressed mice given *L. casei* (S + P).

Table 1
CD4+ and CD8+ lymphocytes and CD11-b+ and CD11-c+ cells in the lamina propria of the small intestine. The number of CD-11b, CD-11c and CD4+ and CD8+ T lymphocytes was determined by direct immunofluorescence of small intestine tissue slides of mice from different experimental groups (normal control mice (NC), normal control mice given the probiotic bacterium in the drinking water (NC + P), stressed control (S) and stressed mice given the probiotic bacterium in the drinking water (S + P)). The results were expressed as the number of positive cells per ten fields of vision (1000×).

Group	CD4+	CD8+	CD11b+	CD11c+
NC	27 ± 4^{a}	33 ± 5^{a}	14 ± 1^{a}	7 ± 2^{a}
NC + P	21 ± 4^{ab}	26 ± 5^{ab}	ND	ND
S	15 ± 5^{b}	21 ± 6^{b}	6 ± 1^{b}	22 ± 5^{b}
S + P	32 ± 11^{a}	24 ± 7^{ab}	20 ± 1^{c}	9 ± 4^{a}

Values are the means for $n = 9 \pm$ SD mice from each group. (a–c) The mean for each cell population without a common letter differs significantly ($p \leqslant 0.05$). ND: None determined.

For lactobacilli populations, we observed that probiotic intake increased the number of lactobacilli in the NC + P and S + P groups. We also observed that the number of enterobacteria was significantly lower in NC + P. There were no differences in lactobacilli and enterobacteria populations in the other groups (Fig. 6D).

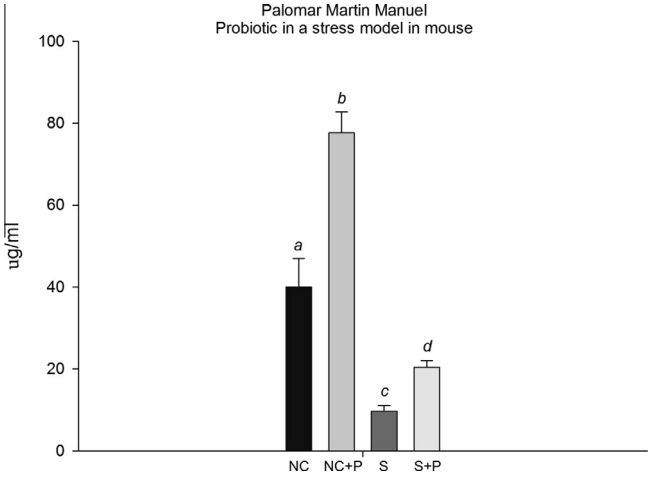

Fig. 4. Determination of secretory IgA concentrations in small intestinal fluid from mice from different experimental groups: Normal control (NC), normal control given *L. casei* (NC + P), stressed control (S) and stressed mice given *L. casei* (S + P). Data correspond to the mean ± SD of results of $N = 9$ animals from three separate experiments. (a–c) Means for each value without a common letter differ significantly ($p \leqslant 0.05$). The figure reveals that stress produced a significant decrease in IgA levels. Administration of the probiotic improved IgA levels to values closer to those of the NC group.

4. Discussion

Recent studies document the existence of a strong association between stress and health, as evidenced by increased susceptibility to disease in a stressed host. The specific mechanisms implicated in this observation are not well understood, but it is undeniable that the immune system is an important target. Various hormones released during stress, have an inhibitory effect on interleukin (IL) IL-12, Tumor Necrosis Factor (TNF-α) and IFN-γ production with increases in the production of regulatory cytokines, such as IL-10, IL-4 and Transforming Growth Factor (TGF-β). This fact partially explains the immunosuppressive effect of glucocorticoids and catecholamines. However, in certain local responses and under specific conditions, stress hormones may exacerbate the inflammatory process through induction of IL-1, IL-6, IL-8, IL-18, TNF-α and IFN-γ, all of which have been implicated in the inflammatory response (Calcagni and Elenkov, 2006).

In our two-factor (food and mobility restriction) stress model, we demonstrated that glucorticoids increased in the stressed group. The probiotic supplementation, having no influence on this parameter, did not appear to act at this level (Fig. 1). Because the NC + P group

had no difference in glucorticoid levels when compared to levels in the NC group (data not shown), these results agree with the fact that the main effect of the probiotic is anti-inflammatory due to a decrease in IL-1 (Zhao et al., 2012; Chen et al., 2009), a cytokine that activates the Hypothalamic–Pituitary-Adrenal axis (HPA) and is responsible for increasing glucocorticoid levels (Goshen and Yirmiya, 2009).

The gut mucosal immune system has mechanisms aimed at avoiding bacterial translocation of the microbiota and maintaining intestinal homeostasis. The intestinal barrier plays a crucial role in mucosal protection. Secretory IgA in the intestinal fluid prevents the adhesion of microorganisms to mucosal surfaces by neutralizing pathogens and binds intestinal bacteria to facilitate elimination through intestinal peristalsis (Brandtzaeg, 2007).

The intact intestinal barrier is maintained by local microbiota, which preserve the architecture of gut villi (Sommer and Backhed, 2013). Stress induces many changes in this barrier and affects the gut histology. In this work, we observed a decrease in the length of villi and a reduction of the crypt depth in the stress group. We observed that this alteration did not occur when stressed mice were given the probiotic bacterium (Fig. 2).

Reduction in the villi length is associated with a decrease in absorption (Xu et al., 2003). In relation to the reduction in crypt depth, we believe that stress induces an increase in the apoptosis of epithelial cells. The improvement observed after probiotic administration may be due to *L. casei* inducing an increase in the expression of the Bcl_2 protein to establish a balance between mitosis and cellular apoptosis, as has been demonstrated in previous work (Perdigón et al., 2002).

Little is known about the neuroendocrine regulation of intestinal IgA; many studies based on experimental models of stress focus on inflammatory processes rather than the mucosal immune response (Maunder, 2005). However, stress does influence the gut humoral response, specifically the production of S-IgA. It has been reported that in a repeated restraint stress model in rats, S-IgA levels in the intestinal mucosa increased significantly as a consequence of stress. This increase may be mediated by norepinephrine, which activates the synthesis of polymeric IgA receptors on the basolateral portion of intestinal epithelial cells, increasing the effect of catecholamines on the transport of polymeric IgA (plgA) across epithelium and release of S-IgA into the lumen (Reyna-Garfias et al., 2010).

Analysis of our results in the mouse stress model showed that the number of IgA+ B-lymphocytes in the lamina propria of the small intestine and the secretion of S-IgA into the intestinal fluid were significantly lower in stressed animals (Figs. 3A and 3B), differing from the results obtained in rat models. However, our results are in agreement with another study that used a mouse stress model (Jarillo-Luna et al., 2007).

An increase in corticosterone can provoke a shift from a Th1 to a Th2 pattern of immunity due to the production of cytokines that promote IgA B cell differentiation (Strober et al., 2005). However, glucocorticoids can also induce a redistribution of lymphocytes in different tissues (Dhabhar et al., 1996; Dhabhar, 2002) and reduce the homing of primed B-IgA+ cells to the mucosa. One possible explanation for the decrease in IgA+ cell numbers in stressed mice could be that high levels of glucocorticoids induce apoptosis of B-cells in the lamina propria of the gut (Fukuzuka et al., 2000; Brunner et al., 2001; Ruiz-Santana et al., 2001). Another possibility could be the low number of CD4+ T cells that produce cytokines such as IFN-γ, IL-6, IL-5, IL-4, and IL-10, which are involved in the expansion and differentiation of IgA+ B cells (Iwakabe et al., 1998; Elenkov, 2004; Brandtzaeg and Johansen, 2005).

In our stress model, we observed that probiotic administration increased the number of IgA producing cells in the lamina propria of the small intestine in stressed mice, but these levels did not reach the number of cells from the NC or NC + P group (Figs. 3A and B 3C and 3D). However, S-IgA did reach a level similar to that of the NC group even when corticosterone levels in serum were not diminished. We think that other factors might be involved in the regulation of IgA+ cell numbers and S-IgA levels.

T cell populations, in particular CD4+ cells, are severely affected by stress; we found that CD4+ cell numbers were restored after probiotic consumption to values similar to those in the NC and NC + P groups. The CD8+ population was diminished in the stress group, but probiotic administration did not influence this cell population (Table 1).

When we analyzed intestinal fluid for IFN-γ levels, we found an increase in the stressed group compared to the normal control (Fig. 5). IFN-γ is important in the synthesis of secretory IgA by intestinal epithelial cells. The probiotic administration reduced IFN-γ levels in stressed mice to values comparable to those in normal control mice, showing that the probiotic restores the physiological status and regulates IgA secretion in some way. Probiotic administration to NC mice induced an increase in IFN-γ levels (Fig. 5).

The most important cells involved in bacterial clearance are macrophages and dendritic cells (DC). We analyzed these cell populations using specific molecule markers expressed on the cell surface, CD11b (macrophages) and CD11c (DCs). These adhesion molecules facilitate the migration of macrophages and DCs to inflammatory sites to remove microorganisms in the infected sites. Another function of these phagocytes is translocating bacteria from the intestinal lumen to maintain intestinal homeostasis.

The stress protocols used in this work induced a decrease in CD11b+ cells and an increase in CD11c+ cells in the lamina propria of the small intestine.

Interestingly, when the mice were given the probiotic bacterium, CD11b+ cell numbers increased significantly compared with those in stressed mice, and expression of CD11c+ cells was restored to normal control values (Table 1).

The results obtained in our stress model mice are in accordance with the results obtained by another study (Tymen et al., 2012), which reported that restraint stress caused a decrease in the number of macrophages and an increase in neutrophil recruitment to infection sites during wound healing. Although we cannot explain

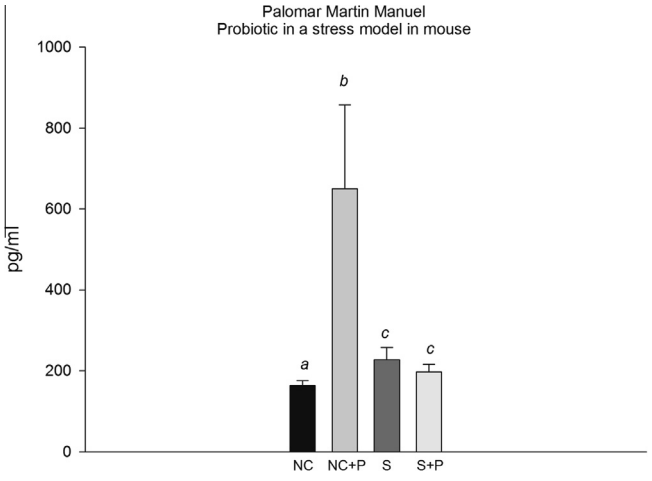

Fig. 5. Determination of IFN-γ concentrations in small intestinal fluid obtained from mice from different experimental groups: Normal control (NC), normal control given *L. casei* (NC + P), stressed control (S) and stressed mice given *L. casei* (S + P). The results are expressed as the mean concentration of IFN-γ (pg/ml) ± SD. Data correspond to the mean ± SD of results for $N = 9$ animals from three separate experiments. Mean values without a common letter differ significantly ($p \leqslant 0.05$). Increases in IFN-γ levels were observed in the NC + P and S groups. Probiotic administration in stressed mice induced a slight decrease in IFN-γ levels.

the increase in CD11c+ cells in our stress group, this effect may be due to regulatory processes that maintain the number of phagocytic cells in the lamina propria of the intestine.

The reciprocal impact of the gastrointestinal tract on brain function has been recognized since the middle of the nineteenth century (Cryan and Dinan, 2012). Recent studies have focused on the impact of microbiota on central nervous system (CNS) function and the influence of stress on the composition of gut microbiota and disturbance of the intestinal barrier (Soderholm and Perdue, 2001).

Studies performed in experimental animals showed that depriving mice of food, water, and bedding produces changes in the stability and composition of the intestinal microbiota, including a decrease in the number of lactobacilli (Tannock and Savage, 1974). Another study conducted in baby monkeys that were separated from their mothers immediately after weaning reported both emotional stress and changes in the intestinal microbiota (Bailey and Coe, 1999).

In our stress model of food and mobility restriction, we did not observe modifications in the different bacterial populations measured (anaerobes, lactobacilli and enterobacteria). The lactobacillus population was significantly increased when a probiotic bacterium was administered to mice, suggesting that the probiotic administration increased the lactobacillus population.

The microbiota balance is important to maintain intestinal homeostasis. Administration of the probiotic to NC animals induced a decrease in enterobacteria and a large increase in lactobacilli.

The microbiota composition and balance among the different populations is extremely important to modulate the host immune system and can influence the host's development and physiology. The key signals to maintain homeostasis and the specific mechanisms by which probiotic bacteria exert beneficial effects on stressed mice are still not well understood (Sommer and Backhed, 2013). We believe that the probiotic bacterium tested in this study

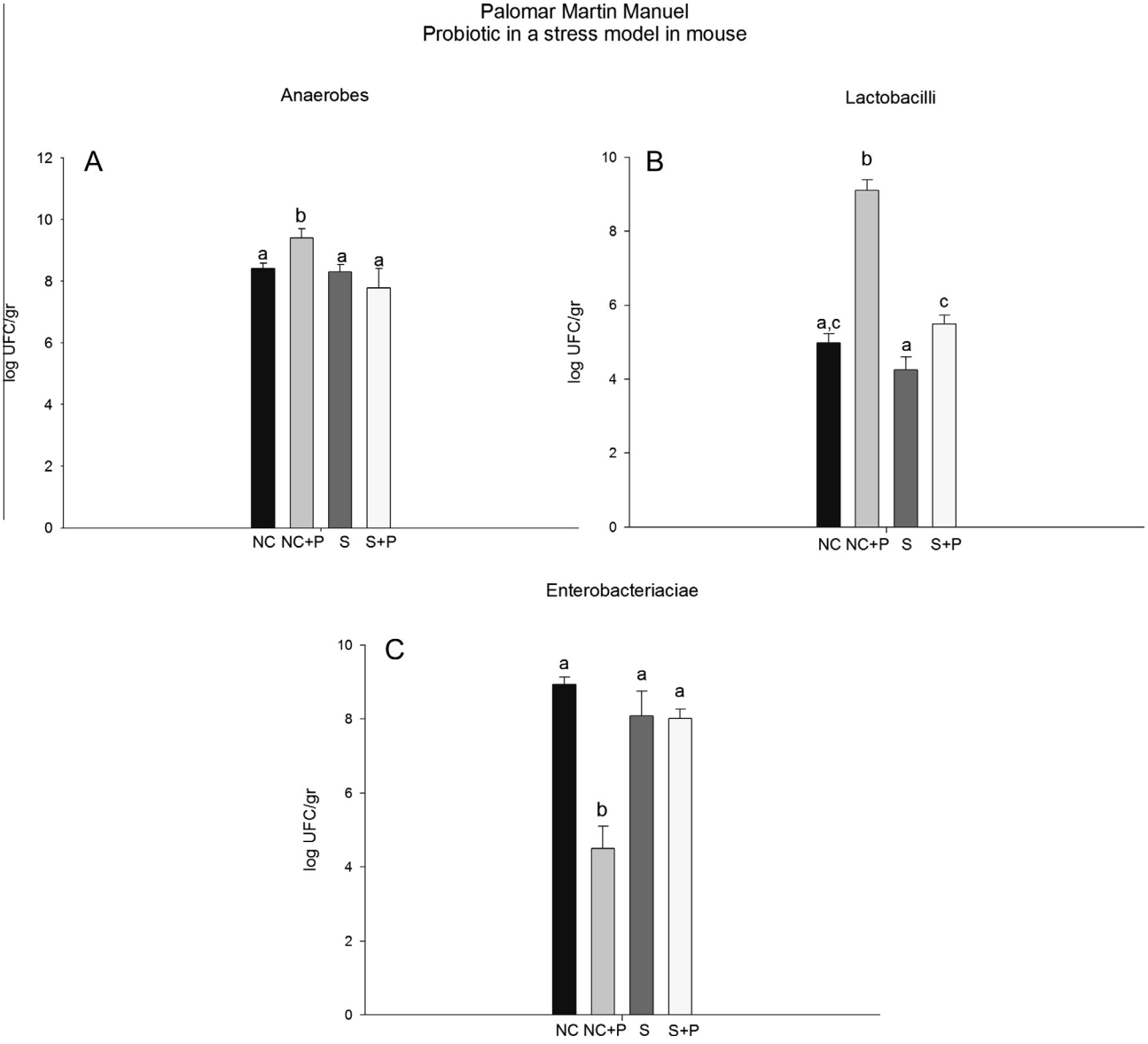

Fig. 6. Large intestines were aseptically removed, weighed and placed in sterile tubes containing peptone water. The samples were immediately homogenized under sterile conditions, serial dilutions of the homogenized samples were performed, and aliquots of the appropriate dilution were spread on the surface of the following agarized media: MacConkey for enterobacteria, MRS for total lactobacilli, and RCA for total anaerobic bacteria. Counts between 20 and 200 colonies per plate were used to determine values. Results were expressed as the log10 bacteria number per gram of large intestine. Each point represents the mean of $n = 9 \pm$ SD. (a–c) Means for each culture medium without a common letter differ significantly ($p \leqslant 0.05$).

exerts a direct effect on the intestinal immune system and regulates intestinal homeostasis and the microbiota balance. Probiotics can also induce changes in cytokine expression (Perdigon et al., 2002) that could affect brain function. This conclusion comes from the observation in our study that probiotic administration did not have an effect on corticosterone levels, which are regulated by the central nervous system (CNS).

We demonstrated that the probiotic *L. casei* CRL 431 repairs some immune parameters affected by stress and improves the microbiota and intestinal ecosystem of the host. This finding leads us to suggest that consumption of food containing this microorganism in stress situations may be a suitable palliative to improve the gut immune system function.

Further studies are being carried out to test our hypothesis that probiotic strains have effects largely through signaling from the probiotics to the gut immune system that lead to improvement of immunity, rather than affecting signals between the brain and endocrine system.

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