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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