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Oral probiotics supplementation can stimulate the immune system in a stress process

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ABSTRACT

In a bifactorial stress mouse model we studied the effect of probiotic supplementation on biochemical parameter in serum, the intestinal impairment of the villi induced by stress, the systemic immune response and the susceptibility to intestinal infections. Probiotic effect was also evaluated on cells from the innate immunity. Mice were subjected to a stress protocol based on food deprivation and movement restrictions for 11 days. We analyzed the probiotic supplementation effect, as probiotic bacteria suspension or as a fermented milk, on the changes induced by stress: biochemical parameters as glucose, triglycerides and cholesterol in serum, body weight and in the gut we determined the number of goblet cells, the length of the microvilli and bacterial translocation from the intestinal microbiota into deep tissues. Peritoneal and spleen macrophages activity, the immune response to ovalbumin immunization, the protection against Salmonella infection during the stress process in mice receiving probiotic, were also determined. We found that probiotic supplementation in the chronic stress model, improves the gut histological structure, increases phagocytic activity of peritoneal and spleen macrophages, enhance the humoral response to the OVA antigen and protect against Salmonella infection. Conclusion: Probiotic supplementation was able to act on the gut and systemic immunity by improvement of the length and cellularity of the villi, increasing the systemic immune response and protecting against Salmonella infection. The effectiveness on the immune system exerted by probiotic consumption suggests the use of these bacteria as an alternative to minimize the damage induced during stress situation.

1. Introduction

Stress condition induces physiological changes affecting the host homeostasis. These changes alter the behavior of the Endocrine and Immune Systems. Different studies have shown that chronic stress causes changes in cellular and humoral immunity [1]. Glucocorticoids and catecholamines released during stress situations also affect the immune functions with an increase in the risk of disease [2,3].

The central nervous system regulates the systemic and mucosal immune response against dangerous environmental stimuli (i.e., physical and psychological stressors) modulating inflammatory or immune response through a complex network of signals from the Nervous, Endocrine and Immune Systems [4].

The microbiota in the gut is a large and stable community as a result of a series of bacterial interactions involving the species best adapted for the given niche [5]. Many studies show that a stressor exposure, can significantly affect the microbiota [6–9]. There are studies showing that mice deprived of food and water suffers changes in the composition of the intestinal microbiota with a decrease in the number of lactobacilli and thereby affecting the intestinal barrier [9,10]. Alterations in the balance of the microbiota increase the translocation from the intestinal lumen to secondary lymphoid organs [11,12]. The increased permeability occurs both in the acute and in the chronic stress and induces bacterial translocation and exacerbation of intestinal inflammation [13–15].

Some studies showed that physiological stressors have impact on the healthy gut of hosts making them susceptible to enteric pathogens [16]. It has been observed that chronic stress reduces the number of peripheral lymphocytes in blood, together with

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reductions in absolute and relative counts of natural killer (NK) and a moderate decreases in T and NK cell function [1,17].

In humans, stress has negative consequences for the body that are associated with risk of cardiovascular and autoimmune diseases, diabetes and infections of the upper respiratory tract [18].

Probiotics are defined as live microorganisms which, when administered in adequate amounts confer a health benefit on the host [19]. They have an important function, in healthy people improving the mucosal immune system by reducing its permeability and enhancing the local immune response, through the increase of secretory IgA [20].

These microorganisms can improve the intestinal barrier influencing the activity of the innate immune cells associated to the gut [21]. Studies performed in healthy mice given *Lactobacillus casei* CRL 431 strain showed that, the consumption of this bacterium, increasing the intestinal barrier and reducing the local inflammatory response against enteric infections and improve the metabolic disorders induced by obesity [22,23].

In a previous work in a bifactorial stress model we determined that, the consumption of *Lactobacillus casei* CRL 431 improved the gut mucosal immune response, increasing immunological parameters such as $CD4^+$ T lymphocytes activation, the number of IgA+ cells as well as the total secretory IgA (S-IgA) released to the intestinal lumen [24].

The aim of the present study was to investigate, the effect of the two probiotic lactobacilli strains consumption, *Lactobacillus (L.) casei* CRL 431 and *Lactobacillus paracasei* CNCMI-1518 and a probiotic fermented milk (PFM), in an experimental model of stress induced by food and mobility restriction in BALB/c mice. We analyzed parameters to reinforce the intestinal barrier, the innate immune response and whether or not probiotic administration can ameliorate the humoral immune response and to protect against an enteric infection.

Our hypothesis is that the consumption of probiotic bacteria or fermented milk containing these microorganisms can improve or minimize the deleterious impact induced by stress on the immune system. This hypothesis is based taking into account the beneficial effect induced by probiotics on the immune system in healthy host.

2. Methods

2.1. Experimental animals

Male BALB/c mice of 5 weeks of age were obtained from the closed random bred colony at CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). Mice 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 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. 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. Probiotic strains and administration protocol

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 into the American Type Culture Collection (ATCC), number 55 544. The other probiotic strain used was *L. paracasei* CNCM I-1518. Both probiotic strains were maintained and controlled at the 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 and *L. paracasei* CNCM I-1518 were administered to mice during stress induction in the drinking water at a concentration of 1×10^8 colony forming units (CFU)/ml, according to standard protocols used in the laboratory [25,26]. The bacteria suspensions were prepared daily to ensure viability and the CFU was controlled to maintain strictly the number of CFU administered every morning at 9:00 am.

Probiotic fermented milk (PFM) contained yoghurt starter cultures (*Lactobacilllus delbrueckii* subsp. *bulgaricus* 10^8 CFU/ml and *Streptococcus thermophilus* 10^8 CFU/ml) and the probiotic bacterium *L. paracasei* CNCM I-1518 (10^8 CFU/ml) was administered to a group of mice replacing the drinking water during all the experience.

Animal's body weights were measured 5 times (on day 1-4-7-10-12) during the experience. The weight of liver, thymus and spleen were taken at the sacrifice (day 12).

2.3. Stress protocols

Stress was induced by two different actors: food deprivation (12 h/day) and movement restriction (3 h/day) during 11 days. This Bi-factorial protocol followed as was described in previous work [24].

2.3.1. Experimental mice groups

Normal Control group (NC) non-stressed group: The animals received balanced diet and water *ad libitum*. Mice of this group were left undisturbed in their home cages.

This non-stressed group was subdivided into 3 subgroups according to the supplementary diet received during the 11 days. For NC + CRL 431 or NC + CNCMI 1518 non stressed mice received a suspension of the probiotic bacteria *L. casei* CRL 431 or *L. paracasei* CNCM I-1518 in the drinking water over the course of the experimental period (11 day). Other non-stressed group received probiotic fermented milk (NC + PFM) during the experimental period.

Stressed group control (S): The animals were subjected (11 days) to the stress protocol. From this group, three subgroups of mice were separated according to the probiotic strains given (*L. casei* CRL 431 or *L. paracasei* CNCM I-1518) or the PFM over the course of the experimental period: (S + CRL431); (S + CNCM I-1518) or (S + PFM) respectively. Each experimental group consisted of three animals for each assay. Each experiment was performed three times.

At day 12, mice from each group were sacrificed by cervical dislocation and samples of serum, intestinal fluid, small intestine, liver, spleen and thymus were taken. Serum and intestinal fluid were stored at −18° C until use. Samples of small intestine taken for the histological studies were processed immediately. Thymus, liver and spleen were weighed and liver and spleen were processed for bacterial translocation studies.

2.4. Biochemical determination

For biochemical determinations the blood collected on day 12, from different groups of mice, was centrifuged at 1000 *g* during 10 min and the serum was separated. Serum glucose, triglycerides (TG), total cholesterol and LDL-cholesterol were determined using enzymatic methods, provided in commercial kits (Wiener Lab., Rosario, Argentina). Results were expressed as concentration of each parameter in the serum (g/l).

2.5. Determination of goblet cells and measurement of the villi length

The small intestine from each experimental and control groups were processed for histological studies according to Saint Marie technique [27]. Then they were deparaffinized and rehydrated in a decreasing gradient of ethanol and incubated in 1% Alcian Blue 8Gx solution (Merck, Darmstadt, F.R. Germany) in 3% acetic acid was used to analyze goblet cells. The histological slides were then incubated with eosin solution and with 0.5% safranin in 0.1 N hydrochloric acid (HCl). They were dehydrated and mounted in Canada balsam (Ciccarelli Lab., San Lorenzo, Argentina). Goblet cells are stained blue. The results were expressed as the number of goblet cells per ten intestinal villi.

In a previous work we observed that the villi lengths in stressed mice were shortened in relation with the non-stressed control and that the probiotic administration improved the histological structure of the villi [24]. To assess this improvement, the length of the villi was measured in the Axiovision software and expressed in μm on the histological slices stained with hematoxylin-eosin. Pictures showing these changes with the length of the villi are shown.

2.6. Determination of the bacterial translocation

We analyzed the possible secondary effects of stress on the intestinal barrier, such as bacteria translocation from the intestine to distant organ as liver or spleen. Animals from each test group were sacrificed on day 12. The organs were aseptically removed, weighed and placed into sterile tubes containing 5 ml of peptone water (0.1%). The samples were immediately homogenized under sterile conditions using a micro-homogenizer (MSE, England). Serial dilutions were made and spread onto the surface of MacConkey, MRS or BHI agar (Britania, Buenos Aires, Argentina) for enterobacteria, lactobacilli and anaerobes respectively. Plates were then incubated in aerobic or anaerobic condition at 37 °C for 24 or 48 h, according the culture medium used. The numbers of CFU in liver or spleen were counted.

2.7. Ex vivo phagocytosis assay of peritoneal macrophages and spleen adherent cells

Peritoneal macrophages and spleen adherent cells from each groups were obtained according to Maldonado Galdeano et al. [28] and the concentration was adjusted at 1×10^6 cells/ml. Phagocytic activity was performed using *Saccharomyces cerevisiae* suspension at a concentration of 10^7 cells/ml previously opsonized using mouse autologous serum (proportion 1:100 of serum: yeast V/V). Equal volumes of opsonized yeast and phagocytic cells were incubated for 30 min at 37 °C. Phagocytic activity was expressed as percentage: in a total of 100 cells counted in optical microscope.

2.8. Systemic immunization protocol

To evaluate the effect of the probiotics under study on the systemic immunity, after stress protocol, animals were divided in 12 experimental groups. All groups (non-stressed, stressed control and stressed with probiotic supplementation) were injected subcutaneously at the end of the stress protocols (day 11), three times every 24 h, with 15 μg of chicken egg albumin (OVA) (Sigma - Aldrich, USA) in phosphate buffered saline (PBS). The mice were sacrificed 10 days after the last OVA injection (day 26). Blood was collected to determine specific anti-OVA IgG in serum by ELISA test. At this time point, peritoneal and spleen macrophages were isolated; the phagocytic activity after immunization was studied. Experimental groups were: a) non-stressed mice immunized with OVA (NC+OVA); b) Normal control with probiotic supplementation (*L. casei* CRL-431 or *L. paracasei* CNCMI1518 or PFM) prior OVA immunization (NC + *L. casei* CRL 431 + OVA) or (NC + *L. paracasei* CNCM I-1518 + OVA) or (NC+PFM+OVA); c) stressed control mice immunized with OVA (S+OVA); d) stressed mice that consumed the probiotics *L. casei* CRL 431 or *L. paracasei* CNCM I-1518 or PFM prior OVA immunization (S + *L. casei* CRL 431 + OVA) or (S + *L. paracasei* CNCM I-1518 + OVA) or (S+PFM+OVA); e) Non-stressed and stressed mice that received continuously (prior and post OVA immunization) the probiotic supplementation during all the experience. They were: (*L. casei* CRL 431 + OVA + *L. casei* CRL 431); (*L. paracasei* CNCM I-1518 + OVA + *L. paracasei* CNCM I-1518); (PFM+ OVA + PFM). For the stressed mice, the supplementation was: (S + *L. casei* CRL 431 + OVA + *L casei* CRL 431); (S + *L. paracasei* CNCM I-1518 + OVA + *L. paracasei* CNCM I-1518); (S + PFM+ OVA + PFM). The experience was repeated three times.

2.9. Determination of specific anti-OVA IgG by ELISA test

The serum samples were collected in all the experimental groups at day 28. The ELISA test was performed using 96 wells microplates that were coated with 1% w/v of OVA solution in carbonate buffer (pH 9.5) and incubated overnight at 4 °C. Non-specific protein-binding sites were blocked with 0,2 ml of PBS containing 0.5% non-fat milk (PBS-milk). Dilutions (1/10000) of the test and control serum samples from the different groups were placed into the PBS-milk and then incubated at room temperature for 2 h. After washing with PBS containing 0.05% Tween 20 (PBS-T), the plates were incubated 1 h with biotin-SP-conjugated goat anti-mouse IgG specific antibody (Jackson Immuno Research Labs Inc, West Grove, USA) and then they were incubated with enzyme concentrated avidin - HRP (BD Bioscience Pharmingen, San Diego, USA) during 30 min. The plates were washed and the TMB reagent (3,3,5,5′ tetramethylbenzidine, BD Biosciences, San Diego, USA) was added and incubated during 20 min. The reaction was stopped with H_2SO_4 (2 N). The absorbance was read at 450 nm using a VERSA Max Microplate Reader (Molecular Devices, USA) and the results are expressed as optical density (OD).

2.10. Determination of the protection against Salmonella enteric serovar Typhimurium challenge

At the end of the stress protocol (day 12) and with the different dietary supplements administered during stress protocol, control and test mice from each group were challenged by gavage with 100 μl/mice of *Salmonella* enteric serovar Typhimurium (*S.* Typhimurium) (1×10^8 cells/ml). The animals were sacrificed 7 days post-challenge, and spleen, liver and large intestine were aseptically removed, and placed into sterile tubes containing 5 ml of peptone water (0.1%). The samples were immediately homogenized and serial dilutions (1/10, 1/20, 1/30 and 1/40) were made and spread onto the surface of MacConkey agar for spleen and liver and Salmonella-Shigella agar (Britania, Buenos Aires, Argentina) for large intestine. The plates were then incubated aerobically at 37 °C for 24 h and the CFU number was counted. The experience was repeated three times.

2.11. Determination of specific anti-Salmonella: IgG in serum and S-IgA in intestinal fluids by ELISA test.

For anti-*Salmonella* S-IgA concentration, intestinal fluids of all the groups were collected on day 7 post-challenge from the small intestines with 1 ml of 0.85% NaCl and immediately centrifuged at 5000 g during 15 min at 4 °C. The supernatant was recovered and ELISA test was performed for anti-*Salmonella* S-IgA. The anti-

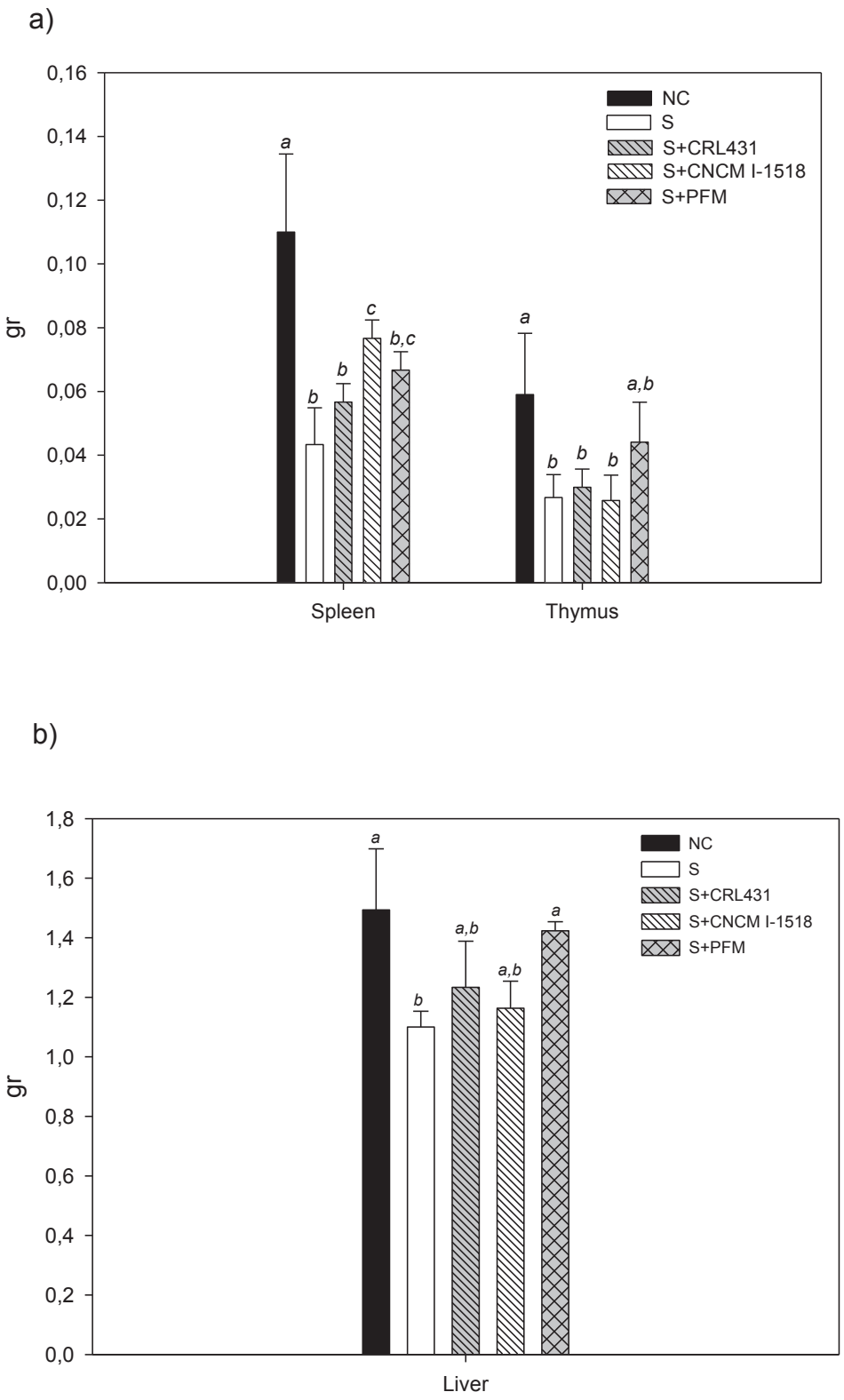

Fig. 1. Changes in the liver, spleen and thymus weight. Spleen, thymus (a) and liver (b) were removed on day 12 and weighed immediately after sacrifice. The weight is expressed in grams. It was observed a decrease in the weight of the three organs in all groups receiving stress protocol. For each trial, each group consisted of 3 mice. Each point represents the mean ± SEM of the weight obtained from the 3 independent trials (N = 9). Student's T test was used to analyze the comparisons between the weights obtained from each test and control group for each time point. Mean values without a common letter differ significantly ($p < 0.05$).

Salmonella IgG, was measured in serum. Blood of mice were collected on day 7 post-challenge, centrifuged at 1000 *g* during 10 min, the serum was recovered and they were diluted 1/8000. Briefly, the plates were coated with 50 μl of a suspension of concentrated and inactivated *S.* Typhimurium suspension (10^{10} CFU/ml) and incubated overnight at 4 °C. Nonspecific protein-

binding sites were blocked with PBS containing 0.5% nonfat milk. Test and control samples from the intestinal fluid and serum were diluted in PBS-milk, added to the plate and then incubated at room temperature for 2 h. After washing with PBS-T, the plates were incubated 1 h with peroxidase-conjugated anti-IgA (Sigma, St. Louis, USA) or Biotin-SP- conjugated anti-mouse IgG (Jackson immunoResearch Baltimore Pike USA). Plates were washed and the TMB reagent was added. The reaction was stopped with H_2SO_4 (2 N). The absorbance was read at 450 nm using a VERSA Max Microplate Reader (Molecular Devices, USA) and the results are expressed as optical density (OD). All determinations were performed in triplicate.

2.11.1. 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 for each determination (no significant differences were observed between individual replicates) ± standard deviation (SD) with $n = 9$. For each immune-histochemical determination and for each mouse, results were obtained from two individual blind counts (by two different researchers).

3. Results

3.1. Changes in the body and organs weight

We observed that the animals that have undergone to stress protocol showed a decrease of 14% in the body weight compared to the normal control group (data not show). This effect was accompanied by a significant decrease in the weight of liver and a lesser extends in the spleen and thymus. The probiotics assayed showed values in the body, spleen, thymus and liver weight near to the stressed mice without significant differences. PFM showed an increase in the thymus weight (Fig. 1a and b).

The ratio body weight/liver between stressed and stressed mice with probiotic or PFM supplementation had a range between in 0,043 to 0,052. The ratio body weight/spleen, the range was 0, 0023 to 0,003 and for the thymus 0, 0011 to 0, 0020.

3.2. Study of biochemical parameters variations in serum

We analyzed the metabolic changes in the serum of stressed mice and given probiotic supplementation. Glucose values showed that probiotic supplementation did not modify the glucose values in the serum of non-stressed mice compared with NC group. Stressed mice showed a significant decrease ($P < 0.05$) in the glucose levels compared to NC groups and the probiotic or PFM administration did not modify these values (Table 1).

Triglycerides decrease slightly in stressed group of mice compared to NC group, without significant differences. The diet supplementation with PFM or probiotic suspensions during stress induction did not have influence in this parameter.

Total cholesterol and HDL-cholesterol concentrations did not showed significant modifications in any of the experimental groups (Table 1).

3.3. Effect of stress and the probiotic consumptions on goblet cells and in the length of the villi

In order to determine if there is any alteration in the histology of the gut that could affect the intestinal barrier during stress situations, we studied the changes in the number of goblet cells mucus producer associated to the intestinal epithelium, which contribute to reinforce the intestinal barrier We also determined the length of the intestinal villi.

The stressed mice showed an important increase in the number of goblet cells compared with the normal control groups. *L. paracasei* CNCM I-1518 strain or PFM administration diminished these values near to the control values (non-stressed group). *L. casei* CRL 431 showed similar effect to those obtained in stressed animals (Fig. 2a).

The length of the intestinal villi was significantly diminished in the stressed mice in relation to the normal control mice ($P < 0.05$). The consumption of any of the two probiotic strains or the PFM, improved villi length reaching similar values to those obtained in non-stressed mice (Fig. 2b). Fig. 2c showed illustrative photographs.

3.4. Bacterial translocation from gut to distant organs such liver and spleen

Stress induce intestinal histological changes could affect the barrier function, allowing translocation of the microorganisms from normal microbiota to distant organs. We investigated the translocation of the normal microbiota to the liver and spleen, which in healthy mice these organs are sterile. We did not find evidence of gut microorganisms translocation to liver or spleen in the animals subjected to bi-factorial stress model (5 groups), as well as in the stressed mice groups given probiotics. The counts of CFU were negative in the organs assayed.

3.5. Determination of the phagocytic activity from peritoneal macrophages and from spleen adherent cells

The phagocytic activity of peritoneal and spleen macrophages were significant decreased in the stressed mice (5 groups) in comparison with non-stressed mice (NC group). Probiotics or PFM supplementation during stress induced significant increases in the phagocytic activity of peritoneal and spleen macrophages as regard to the stressed control, reaching values near to the controls (Fig. 3a).

Table 1
Biochemical parameters in blood serum.

	NC	NC+CRL431	NC+CNCM I-1518	NC+PFM	S	S+CRL 431	S+CNCM I-1518	S+PFM
Glucose	152,00 ± 32,91[a]	184,50 ± 44,55[a]	189,00 ± 17,00[a]	200,67 ± 17,01[a]	93,01 ± 25,54 [b]	107,00 ± 24,04[a]	66,00 ± 15,62 [b]	119,67 ± 18,77[a]
Cholesterol	115,00 ± 12,12[a]	115,00 ± 9,90[a]	124,67 ± 8,14[a]	126,67 ± 13,01[a]	97,00 ± 11,79[a]	97,00 ± 9,90[a]	96,67 ± 6,16[a]	102,67 ± 9,45[a]
Triglycerides	199,33 ± 85,82[a]	39,60 ± 36,90 [b]	205,33 ± 5,13[a]	240,00 ± 30,79[a]	137,00 ± 7,07[a]	151,00 ± 43,84[a]	160,67 ± 20,03[a]	133,67 ± 15,04[a]
HDL	72,67 ± 12,66[a]	75,50 ± 0,71[a]	77,67 ± 1,53[a]	68,67 ± 8,08[a]	54,33 ± 12,50[a]	59,00 ± 12,73[a]	64,67 ± 1,15[a]	91,67 ± 7,66[a]

Biochemical parameters were measured on day 12, in the serum of mice from different experimental groups: Normal control (NC); Normal control plus L. casei CRL 431 (NC+CRL431) or L. paracasei CNCM I-1518 (NC+CNCM I-1518) or with PFM (NC+PFM). Stressed mice (S); stressed mice that received the probiotic strains (S+CRL 431 or S+CNCM I-1518) or the PFM (S+PFM). Results were obtained using enzymatic methods and were expressed as g/l. Values are the means for $N = 9$ mice ± SD obtained in each group (from 3 independent experiments). [a,b] Means for each clinical parameter without a common letter differ significantly ($P < 0.05$).

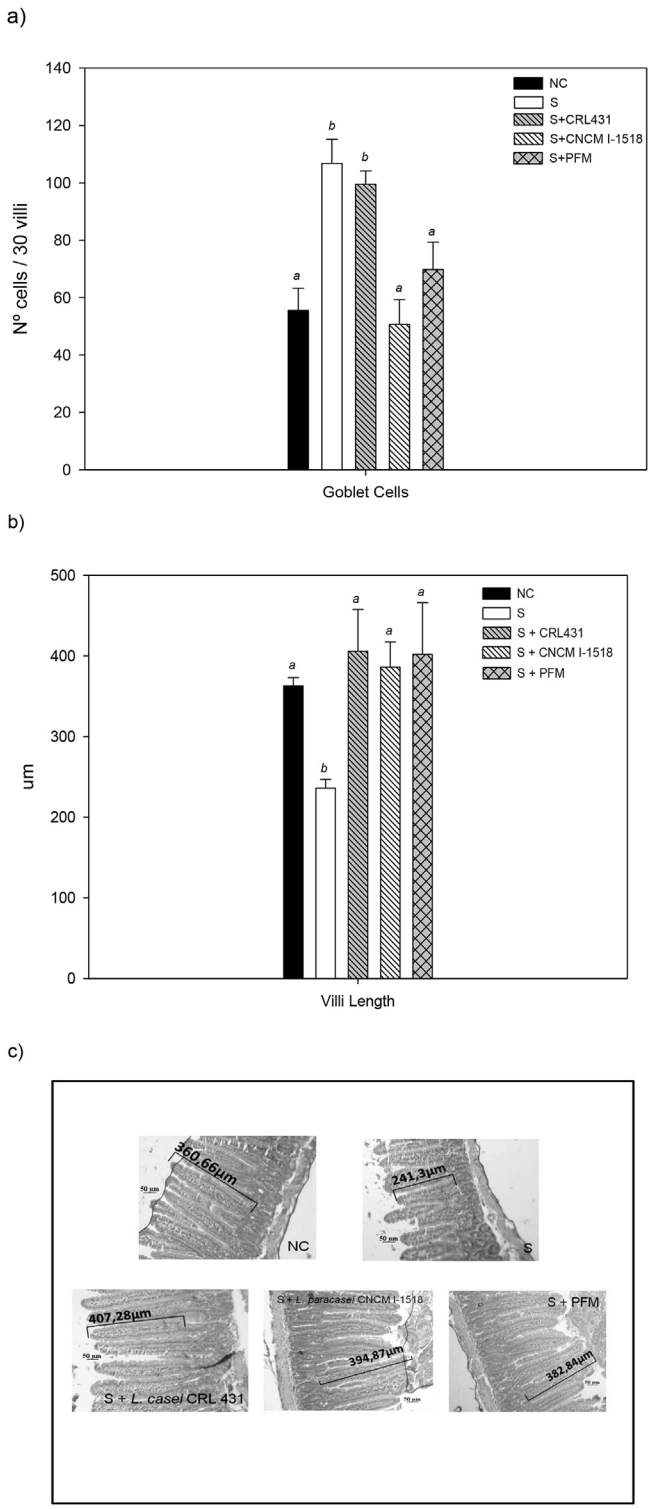

Fig. 2. Number of goblet cells and length of the intestinal villi. a). The number of goblet cells was measured in all groups on day 12. The amount of these cells was counted in serial sections of small intestine. It was conducted in a total of 30 villus per intestinal section analyzed. The figure showed increases in the amount of goblet cells in animals of groups S and S + *L. casei* CRL431 and a decrease in the normal values for group S + *L. paracasei* CNCM I-1518 and S + PFM. b). The villi length was measured in serial sections of the small intestine stained with hematoxilin-eosin. The AxioVision software was used to measure the length of villi and it is expressed in microns. The groups of stressed animals receiving the diet with probiotics showed no change in the length of small intestinal villi compared to the control group. Data correspond to the mean ± SD of results of N = 9 animals from three separate (a,b) Means for each value without a common letter differ significantly ($p < 0.05$). c) Histological study. Show representative pictures from slices of the small intestine of mice after staining with hematoxilin-eosin. The samples were obtained at 12 days of experience with a magnification of 100 X. a) NC group; b) S group: the intestinal villi are shorter than in the NC group. The probiotics or PFM (c, d and e) administration improved the histological modification caused by stress.

3.6. Phagocityc activity of peritoneal macrophages and spleen phagocytes after OVA immunization

After OVA immunization we observed that the activity of peritoneal macrophages was significantly lower in the stressed mice (S+OVA) than in the control group of mice (NC+OVA). The previous or continuous probiotics treatment showed a slight improvement with values near to the NC group. The results obtained with spleen phagocytes showed that their phagocytic activity was diminished in the stressed mice immunized with OVA (S+OVA). This parameter was recovered in macrophages from stressed mice that received *L. casei* CRL 431 and PFM prior to OVA immunization. Continuous probiotics administration was not effective to increase the phagocytic activity of the spleen macrophages. These results are expressed in Fig. 3b.

3.7. Effect on the systemic antibody response

The humoral immune response to the ovalbumin antigen was analyzed by the specific anti OVA antibody production. We observed in non-stressed mice, the probiotics or the PFM administration did not showed significantly increases in the levels of specific anti-OVA IgG. In stress condition, the specific immune response was affected, being the levels of the anti-OVA IgG significantly lower ($P^{<}$ 0.05) than the levels obtained in non-stressed control mice. *L. paracasei* CNCM I-1518 administered previous or continuously to OVA immunization protocols, was the only probiotic that had an adjuvant effect, reaching to normal control values (Fig. 4).

3.8. Salmonella infection and determination of anti-Salmonella IgG and S-IgA in stressed mice

We analyzed if the probiotics supplementation in stressed mice was effective to protect against *Salmonella* Thyphimurium infection. Translocation of pathogen to liver, spleen and the colonization in the large intestine, was determined, as well as, specific IgG in serum or S-IgA in the intestinal fluid. We observed in the non-stressed and infected mice given any probiotic supplementation a significant decrease in the number of CFU in the organs assayed, being the PFM administration more effective to protect against the pathogen in comparison with the infection control (NC + Sal.). In the stressed mice infected group given *L. paracasei* CNCM I-1518 or PFM we found that the number of CFU in large intestine, liver and spleen were significantly diminished. *L. casei* CRL 431 was not effective in the protection against Salmonella in stressed host (Fig. 5 a, and b).

We determined that the stress conditions impair the specific humoral immune response to Salmonella. Low levels of anti-Salmonella IgG were found in serum in comparison with the non-stressed mice (NC + Sal.). The administration of the probiotic strain *L. casei* CRL 431 or the PFM increased these values near to the values of non-stressed mice infected with *Salmonella* (Fig. 6).

The specific anti-Salmonella S-IgA in intestinal fluid did not increase in the stressed group in relation with the non-stressed group (NC + Sal). The level of S-IgA in the group given *L. casei* CRL431 remained in similar levels to the non-stressed control group. L. paracasei CNCM I-1518 supplementation increased the

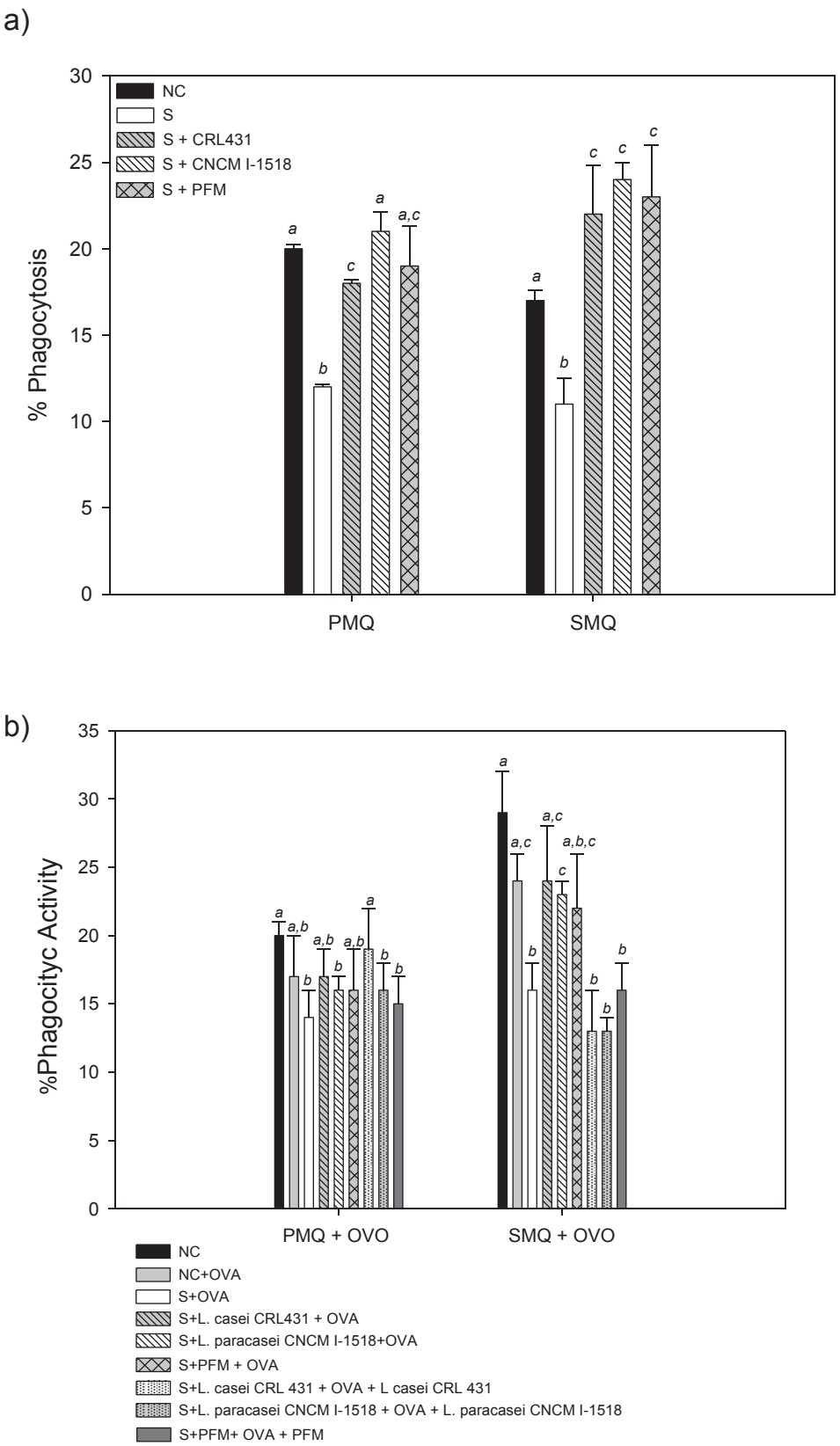

Fig. 3. Phagocytic activity of peritoneal and spleen macrophages. **a).** Phagocytic activity of macrophages isolated from peritoneum (PMQ) and spleen (SMQ) were analyzed. Mice were sacrificed at day 12 to isolate the macrophages from peritoneum and spleen. The values for phagocytic activity were expressed as mean for N = 9 ± SD of percentage of phagocyting macrophages in 100 cells counted. a,b,c,d,e,f Means values without a common letter differ significantly ($P < 0.05$). b). Phagocytic activity of macrophages after OVA immunization. Phagocytic activity of macrophages isolated from spleen and peritoneum were analyzed. Mice were given probiotic strains or PFM prior OVA inoculation. Other group received probiotic consumption during all the immunization time. They were sacrificed at day 10 after the 3rd OVA injection to isolate the macrophages from peritoneum and spleen. The values for phagocytic activity were expressed as mean for N = 9 ± SD of percentage of phagocyting macrophages in 200 cells counted. a,b,c,d,e,f Means values without a common letter differ significantly ($P < 0.05$).

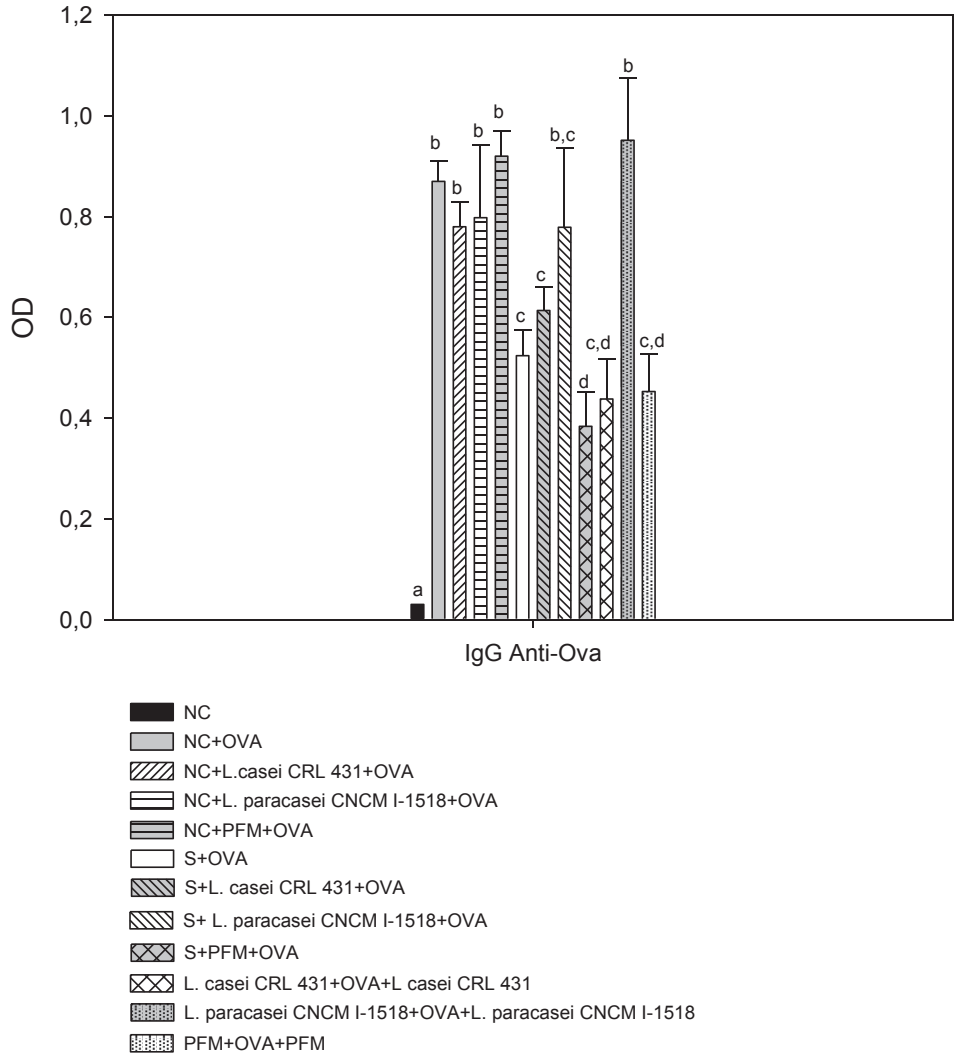

Fig. 4. Specific anti-OVA antibodies. Specific anti-OVA IgG was determined in blood serum by ELISA test in the samples obtained 10 d after the 3rd OVA injection. Results are expressed as OD (450 nm). Each bar point represents the mean of N = ± SD mice from each group. a, b, c, d means values without a common letter differ significantly (P < 0,05).

values obtained in the stressed control group, as well as, the PFM administration in relation with the infected normal and stressed control groups (Fig. 6).

4. Discussion

The gastrointestinal tract and the immune system are particularly sensitive to different stressors agents. In a previous study using a mouse model, we demonstrated that, the stress induced changes in the gut Mucosal Immune System. We determined that the probiotic strain *L. casei* CRL 431 supplementation improves the gut mucosa immunity [24].

In the present study, we analyzed if the stress situations had influence on biochemical parameters such as glucose, HDL, LDL cholesterol serum levels. We also studied some parameters of the intestinal barrier as the mucus producing cells (goblet cells), the villi length, gut bacteria translocation from the intestinal microbiota to internal organs and the influence of probiotic supplementation on these parameters. The activation of the cells from the innate immune response such as peritoneal and spleen macrophages and the antibody response to ovalbumin, were determined. The probiotic administration as a palliative to protect against *Salmonella* infection under stress condition was also investigated.

Studies conducted in healthy human undergoing long periods of stress showed a significant increase in abdominal adipose tissue and early signs of metabolic syndrome, suggesting that stress play an important role in the onset of metabolic abnormalities [29], as well as changes in the body weight during stress period.

Other studies showed that the chronic stress induced by food restriction produce a deregulation in the insulin and leptin action with reduction in the blood levels of both hormones that affect biochemical parameters [30,31].

In our experimental model, the restraint stress induced a decrease in the glucose levels in blood; this parameter was not improved in mice that received the probiotic bacteria or the PFM. The lipid profile (triglycerides and cholesterol levels) did not showed significant changes among the different groups. The results obtained show that the probiotics or PFM supplementation during stress, do not affect these parameters, mainly in the lipid profile (Table 1).

We observed a decrease in the body and organs weight. These results do not agree with a report that demonstrated that chronic stress induces physiological and neuroendocrine changes associated with an increased food intake and adipogenesis [32]. Our differences in the body weight could be explained because the animals are deprived of food ingestion during the dark cycle, which

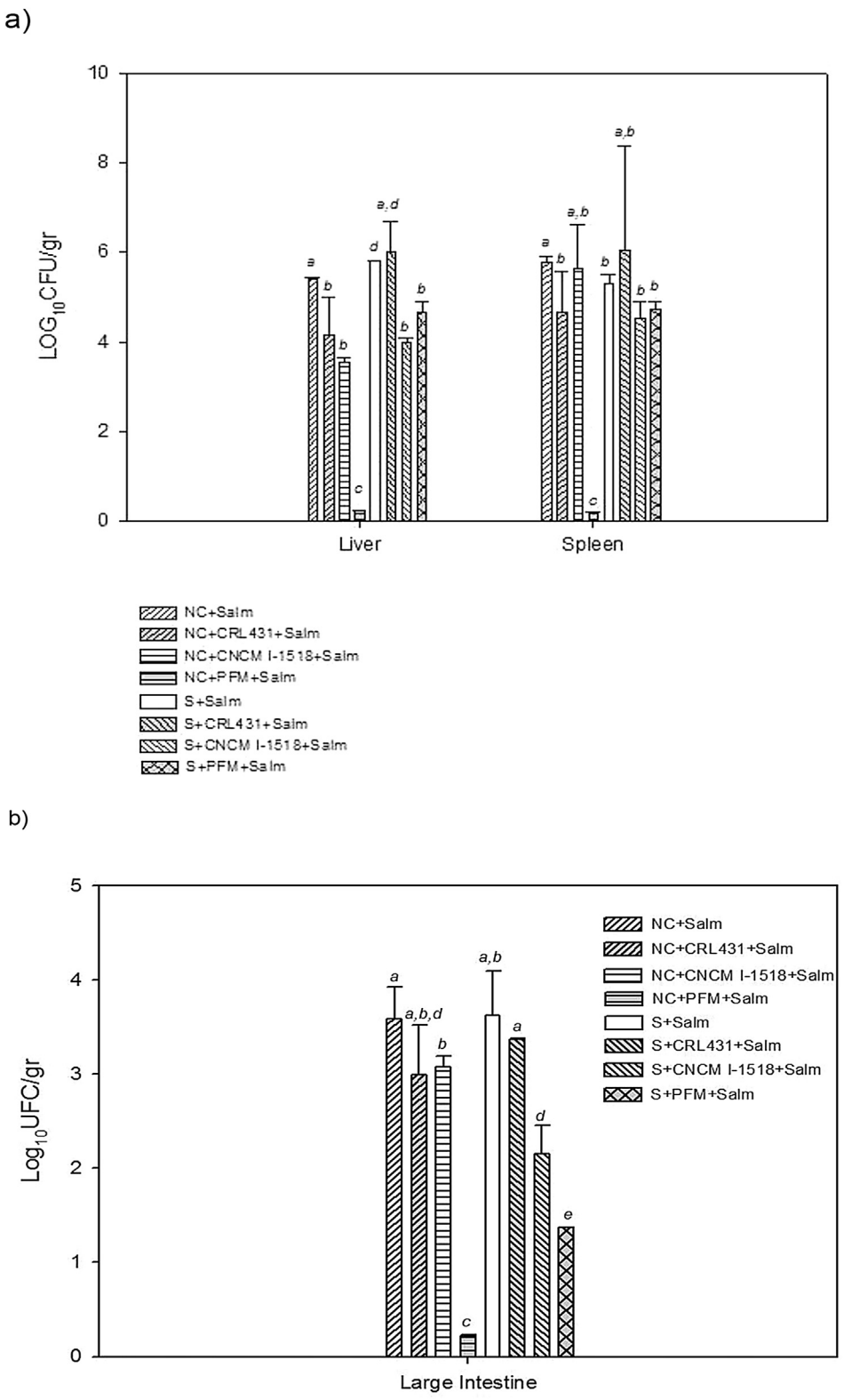

Fig. 5. Salmonella colonization in liver, spleen and large intestine. Spleen, liver and large intestines, were aseptically removed, weighed and placed in sterile tubes containing peptone water. The samples were immediately homogenized under sterile conditions, serial dilutions of the homogenized samples were performed, and aliquots of the appropriate dilution were spread on the surface of following agarized media (a) Mac Conkey for spleen and liver or (b). Salmonella-Shigella for large intestine. Counts between 20 and 200 colonies per plate were used to determine the value ranges. Results were expressed as the log10 bacteria number per gram of large intestine. Each point represents the mean of $n = 9 \pm SD$. (a–f) Means for each culture medium without a common letter differ significantly ($p < 0.05$).

is the period of major physical and feeding activity of rodents, following to the restraint period during the light cycle.

Reinforcing our findings, several investigators attributed that stress induces anorexia by corticotropin-releasing hormone (CRH)

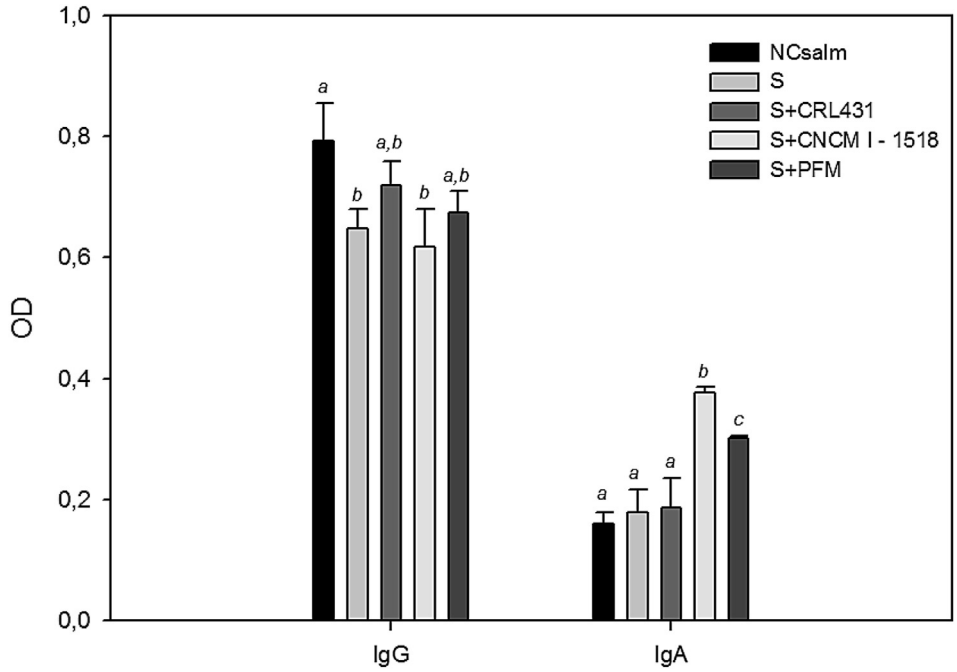

Fig. 6. Anti-Salmonella IgG and S-IgA. Anti-Salmonella IgG and S-IgA were determined in blood serum for IgG and intestinal fluid for S-IgA by ELISA test in the samples obtained after sacrifice. Results are expressed as OD (450 nm). Each bar point represents the mean of N = ± SD mice from each group.

and/or serotonin (5-hydroxytryptamine, 5-HT) pathways [16]), involved in the regulation of feeding behavior, like depression of appetite [33,34]. It was demonstrated that the restraint stress has different effects on feeding behavior and in the body weight depending on the different moments of the light-dark cycle in which the stress is applied [35]. This fact could explain the lower glucose levels and the unalterable levels of triglycerides and cholesterol, as in the body weight. These were also explaining the decrease in the liver, spleen and thymus weight found in our stressed model. Probiotic or PFM supplementation in the stress model did not induced changes on the blood parameters neither in the body weight, probably because the appetite is regulated by hormones, the probiotics do not act at this level, as was demonstrated in a previous study, where the probiotic administration did not have an effect on the corticosterone levels [24]. Neither the supplementation assayed had an effect on the body weight. A slight increase in the liver and spleen weight was observed, and only the PFM increased thymus weight (Fig. 1).

Stress impaired the histology of the intestine, inducing shortening of the villi length and increasing the number of goblet cells, implicated in the secretion of mucus. The mucus is a protective layer covering the intestinal epithelial cells (IECs). This layer acts as an active defensive barrier, maintaining the intestinal microbiota physically separated of the IECs [36]. Perhaps this is the reason why we did not observed microbiota translocation to internal organs. Probiotic supplementation stabilized the number of goblet cells with values near to the non-stressed control (Fig. 2a).

Studies performed in a non-severe protein-energy-malnutrition model in mice showed that the intestinal villi in malnourished mice were shorter compared with the well nourished group. In this malnutrition model, the mice re-nourished for 5 days with a PFM, recovered the normal architecture of the intestinal villi [23].

In our stress model, we found that the type of stress applied acts synergistically with the restraint period and induced changes in the length of the intestinal villi. The importance of the villi length in the gut function is well demonstrated in malnutrition where the intestinal absorptive capacity decreased via shortening of villi [37,38]. Probiotics or PFM supplementation to stressed mice recovered the normal architecture and the length of the intestinal villi, which favors the absorption and the normal function of the gut, as well as the signals to stimulate the immune cells associated to the gut, with an improvement in the immune response. Our results showed an improvement in the length of the villi induced by the probiotics assayed. This fact is very important to maintain the intestinal homeostasis (Fig. 2b and c).

Macrophages are a key cell type in the immune system, which play crucial roles in innate immunity and can respond to local immune- and/or pathogen-derived signals [39]. It was known that glucocorticoids are important regulators of the immune system and the phagocytic cells are sensitive to glucocorticoid action [40]. Studies performed by Lim et al. 2007 demonstrated that the macrophages function is dependent of the glucorticoid concentration, so low amounts of corticosterone enhances immune functions and high concentrations of glucocorticoids repress macrophage function which would be harmful to the organism [41]. Corticosterone is induced in stress situation, when we determined the phagocytic activity of peritoneal and spleen macrophages we observed that the function of these cells was diminished, and that the probiotic bacteria administration allowed to normalize the phagocitic activity of peritoneal and spleen macrophages (Fig. 3a). We evaluated the macrophages function and the specific IgG levels after OVA immunization, we observed that the two probiotic strains assayed were able to normalize the phagocytic activity of peritoneal macrophages, but only *L. paracasei* CNCM I-1518 had similar effect in the spleen macrophages (Fig. 3b). For the specific anti-OVA-IgG level in serum from stressed mice that consumed the probiotic strain *L. paracasei* CNCM I-1518 prior and after immunization was the supplement more effective to improve the humoral immune response (Fig. 4).

It is known that stress impair the, course of an infection, mainly due to the immunosuppressive effect provoked by the hormones induced during stress that affect the immune system cells behavior [42].

We analyzed the response of stressed mice against *S.*

Thyphimurium infection. The results showed in stressed mice, challenged with *Salmonella* a high number of UFC in the organs assayed compared with the non-stressed group. In animals that received probiotic supplementation, we observed that the PFM containing *L. paracasei* CNCM I-1518 was the most effective in the protection against Salmonella infection (Fig. 5a and b). These results agree with the values for the anti-Salmonella IgG or S-IgA found, indicating that probiotic supplementation was able to protect against the pathogen through an increase in the adaptive immune response (Fig. 6).

5. Conclusion

We demonstrated in a stress model in mouse, a diminution in the glucose levels in blood serum and loss in the body weight. The main target of this stress protocol is the immune system, with impairment in the behavior of the innate immune cells; being the macrophages the most affected cells with humoral immunity deterioration against OVA or an enteropathogen, due to the macrophages, are key cells in the adaptive immune response induction.

We demonstrated that the probiotic supplementation reversed the deficiencies in the immune response induced during the stress improving the architecture of the small intestine, avoiding the bacterial translocation from the intestine to deep tissues and reinforcing the intestinal barrier. The probiotics assayed improved the phagocytic activity of peritoneal and spleen macrophage, the antibody response to OVA immunization and improved the protection against *Salmonella* infection. The present results are an important contribution in the demonstration for the safety of probiotic supplementation in stress conditions and in the improvement of the immune response. This fact allows proposing the use of selected probiotic strains or PFM as adjuvants of the gut mucosal and systemic immune response under stress situation.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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