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# ADJUVANT EFFECT OF A PROBIOTIC FERMENTED MILK IN THE PROTECTION AGAINST *SALMONELLA ENTERITIDIS* SEROVAR *TYPHIMURIUM* INFECTION: MECHANISMS INVOLVED

A. DE MORENO DE LEBLANC[1], C. MALDONADO GALDEANO[1,2], C.A. DOGI[1], E. CARMUEGA[3], R. WEILL[4] and G. PERDIGÓN[1,2]

[1]*Centro de Referencia para Lactobacilos (CERELA-CONICET), Tucumán;* [2]*Instituto de Microbiología. Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán;* [3]*Nutritia, Buenos Aires;* [4]*Departamento de investigación y Desarrollo, DANONE Argentina S.A. Buenos Aires, Argentina*

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**Probiotics may offer protection against *Salmonella enteritidis* serovar *Typhimurium* infection via different mechanisms. The aim of this study is to investigate, using mouse models, the effect of the administration of fermented milk containing the probiotic bacteria *L. casei* DN-114 001 in the protection against *Salmonella enteritidis* serovar *Typhimurium* when this product is administered continuously before and after infection or only post-infection. The adjuvant effect of this probiotic fermented milk (PFM) against *S. Typhimurium* was also evaluated in newborn mice, whose mothers received the PFM during the suckling period or their offspring after weaning. The results obtained showed that PFM administration after salmonella infection was useful to decrease the severity of the infection. The best effect was obtained with continuous PFM administration. In the newborn mice model, PFM administration to the newborn mice after weaning showed the best effect against the pathogen. PFM administration to the mother during the suckling period was beneficial against this enterophatogen when their offspring did not receive probiotics after weaning. Continuous PFM administration to adult mice (before and after infection) was important to maintain the intestinal barrier and the immune surveillance in optimal conditions to diminish the pathway of entrance of salmonella and the spread of this pathogen to deeper tissues. In the newborn mice model, it was observed that PFM administration to the offspring after weaning or their mother during the suckling period had a protective effect against salmonella infection, however, in the mice from mothers that received PFM during nursing which were fed with PFM after weaning, we found a down regulated immune maturity that was not protective against this infection.**

Lactic acid bacteria (LAB) are found in large numbers in the gut of healthy animals and they have received much attention in the past decades for their use as probiotic microorganisms traditionally used in food fermentation (1). For these and other reasons, there is a steady increase in the consumption of fermented dairy products (i.e. yoghurt and other fermented milks) containing viable LAB and they are included in daily diets.

Gastroenteritis is the most frequent cause of

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*Mailing address*: Dr Gabriela Perdigón,
CERELA-CONICET,
Chacabuco 145,
San Miguel de Tucumán.
Tucumán. (T4000ILC), Argentina
Tel: ++54 381 4310465 Fax: ++54 381 4005600
e-mail: perdigon@cerela.org.ar

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acute diarrhea and studies have been carried out to investigate the effects of probiotics on diarrhea caused by different pathogenic agents (2-6). Salmonellosis is one of the most common bacterial infections in the world. It is responsible for a variety of clinical syndromes, including enteric fever (usually caused by typhoid or paratyphoid species), enterocolitis, bacteremia, and severe local infections. Probiotics may offer protection against salmonella infection via different mechanisms (7). *In vitro* and *in vivo* studies with animal models are used to learn more about the possible mechanisms involved in the prevention of this pathogen by probiotic bacteria. In this sense, the pre-treatment of a human epithelial cell line with *Lactobacillus* or *Bifidobacterium* resulted in inhibition of salmonella and induced IL-8 expression (8). Furthermore, treatment of mice with a fermented milk containing *B. longum* led to a decrease of the pathogenesis of *Salmonella enteritidis* serovar *Typhimurium* (*S. Typhimurium*) infection may be due to a reduced inflammatory response (9).

There are many reports on the beneficial effect of the consumption of fermented milk containing the probiotic strain *L. casei* DN-114 001. It was observed that long-term probiotic fermented milk (PFM) administration had immunodulatory effects and maintained the intestinal homeostasis without any adverse secondary effects in mice (10). The administration of this PFM beneficially affected the intestinal microbiota and gut associated immune cells of nursing mice and after weaning until immune maturity (11).

The aim of this study is to investigate, using mouse models, the adjuvant effect of the administration of a fermented milk containing the probiotic bacteria *L. casei* DN-114 001 in the protection against *S. Typhimurium* as well as the mechanisms involved in this protective effect when the product is administered continuously before and after infection or as a treatment (after infection). The adjuvant effect of this PFM against *S. Typhimurium* infection in the early period of life was also evaluated in newborn mice, from mothers that received the PFM during the suckling period or their offspring after weaning.

## MATERIALS AND METHODS

### *Animals and bacterial strains*

The BALB/c mice used in this study were obtained from the closed random-bred colony maintained at the CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). All the animals were housed in cages kept in a controlled atmosphere (22 ± 2ºC; 55 ± 2% relative humidity) with a 12 h light/dark cycle, and fed *ad libitum* with a conventional balanced diet.

The studies were carried out with adult and newborn mice. A) Adult mice were five to six weeks old weighing 22-26g. B) Pregnant mice (7 weeks old weighing 25-30g) were identified and monitored daily until delivery. The day of birth was identified as day 0 of life. Babies were weaned at 21 days of age. At weaning, the offspring were separated from their mothers.

*Salmonella enterica* serovar *Typhimurium* strain was obtained from the Bacteriology Department of the Hospital del Niño Jesus (San Miguel de Tucuman, Argentina). For each pathogen, an aliquot (200 µl) from an overnight culture was placed in 5 ml of sterile BHI broth and incubated for 4 h. The concentration of bacteria was adjusted to $1x10^8$ or $1x10^6$ CFU / ml in phosphate buffered saline (PBS), for adults or newborn mice, respectively.

### *Feeding with probiotic fermented milk and infection procedures*

Commercial fermented milk containing the yogurt starter cultures (*L. delbrueckii* subsp. *bulgaricus* $10^8$ CFU/ml and *Streptococcus thermophilus* $10^8$ CFU/ml) and the probiotic bacterium *L. casei* DN-114 001 ($10^8$ CFU/ml) was used in this study. In all the experimental design the animals received this probiotic fermented milk (PFM) *ad libitum*.

Three experimental groups, of 9 animals each, were analyzed for the study with adult mice and *S. Typhimurium* infection: 1) For the continuous administration of the PFM, mice were given PFM during five days, challenged with *S. Typhimurium* and continued receiving the PFM during 7 days post challenge. 2) For the effect post-infection, mice were challenged with *S. Typhimurium* and the PFM was added to their daily diet post infection during 7 days. 3) The infection control group did not receive special feeding.

All the groups were challenged with 100 µl of $1x10^8$ CFU / ml of *S. Typhimurium* ($LD_{50}$) by oral administration. Animals were sacrificed 2 and 7 days post-challenge (3 animals each day).

Fig. 1 shows the experimental design for newborn mice infected with *S. Typhimurium*. The protocol contained two experimental groups of mothers. We used 7 mothers: 3 received PFM during the suckling period (B) and 4 did not (A). At weaning (21 days of age), in both groups of mothers, the offspring were divided into two subgroups: (b) those receiving PFM and (a) those that did not. Twelve offspring were in each group. They were the groups Aa, Ab, Ba and Bb. At day 28, all the mice were challenged

with the enterophatogen bacteria (S). After the infection, mice from groups Ab and Bb continued receiving the PFM, similar to the adult mice with continuous PFM before and after challenge, until the sacrifice (day 35); and mice from the group Aa and Ba were divided in two subgroups: Those that received PFM, and those did not.

All the groups were challenged with 100 µl of $1x10^6$ CFU / ml of *S. Typhimurium* by oral administration. The offspring were sacrificed 7 days post-challenge to obtain the samples. The mice were weighed throughout both experiments (adults and newborns) and the number of deaths was registered. For all the experiments the values (N=9) were the means of 3 independent trials (3 mice were sacrificed for each sample and trial). All animal protocols were pre-approved by the Animal Protection Committee of CERELA and all experiments comply with the current laws of Argentina.

*Colonization assays*

The spleen, liver and large intestine 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 microhomogenizer (MSE, England). Serial dilutions were made and spread onto the surface of MacConkey agar (Britania, Buenos Aires, Argentina) for liver and spleen and salmonella- shigella agar (Britania, Buenos Aires, Argentina) for large intestine samples. The plates were then incubated aerobically at 37°C for 18 h.

*Determination of total and specific IgA in intestinal fluids*

Intestinal fluids were collected from the small intestines of adults or offspring mice with 1 ml of 0.85% NaCl and immediately centrifuged at 5000 *g* for 15 min at 4°C. The supernatants were recovered and stored at -20°C until IgA determination.

ELISA was used to measure the concentration of total S-IgA according to the technique described by LeBlanc et al. (12). Briefly, affinity-purified monoclonal goat anti-IgA (α-chain specific Sigma, St Louis, MO, USA) was added in 0.05 M carbonate-bicarbonate buffer (pH 9.6) to 96 wells plates and incubated at 37°C for 1 h. The plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked for 1 h at 25°C with 0.5% non-fat dry milk in PBS. Plates were washed and incubated for 2 h at 37°C with either 50 µl of standard kappa IgA (Sigma, St. Louis, USA) or 50 µl of intestinal fluid samples. Plates were washed and incubated in the presence of horseradish peroxidase-conjugated anti-IgA-specific antibodies (Sigma, St. Louis, USA) for 1 h at 37°C, after which the plates were again washed and trimethylbenzidine (TMB) reagent containing peroxide (BD Biosciences, San Diego, USA) was added. 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).

For the specific antibody determinations, plates were coated with 50 µl of a suspension of concentrated and heat-inactivated *S. Typhimurium* ($>10^{10}$ UFC/ml) and incubated overnight at 4°C. Non-specific protein-binding sites were blocked with PBS (pH 7.2) containing 0.5% non-fat milk. Dilutions (1/50) in PBS of the test and control samples from the intestinal fluid were then incubated at room temperature for 2 h. After washing with PBS-T, the plates were incubated for 1 h with peroxidase-conjugated anti-IgA-specific antibodies. Plates were again washed, the TMB reagent was added and the reactions were stopped with $H_2SO_4$ (2 N). The absorbance was read at 450 nm.

*ELISA assays for cytokines in intestinal fluids*

Intestinal fluids were collected as explained above and to determine the concentration of the cytokines (IFNγ, IL-10), BD OptEIA™ mouse cytokine ELISA kits from BD Bioscience (San Diego, USA) were used. The results are expressed as concentration of each cytokine in the intestinal fluid (pg/ml).

*Isolation of macrophages from peritoneum and Peyer's patches of adult mice. Determination of microbicidal activity*

Macrophages from peritoneal cavity and Peyer's patches were obtained from adult mice after five days of PFM administration or two days after challenge with *S. Typhimurium*. Peritoneal macrophages were obtained according to Valdez et al. (13). Macrophages were extracted from the peritoneal cavity with 5 ml of sterile PBS, pH 7.4 containing 100 µg/ml of gentamicin (Gm). For the isolation of macrophages from Peyer's patches, the protocol described by Maldonado Galdeano et al. (14) was used. The small intestine of each mouse was removed, washed and the Peyer's patches were excised in Hank's buffered salt solution (HBSS) containing 4% foetal bovine serum (FBS). The epithelium cells were separated with an HBSS/FBS solution containing EDTA. The sediments were incubated with dispase/DNase solution and the mononuclear cells were recovered. These cells were collected from the supernatant and washed with RPMI 1640 medium (Sigma, St. Louis, USA). The macrophages were separated from the whole mononuclear population using their adherence property on glass slides.

Microbicidal activity was determined with the technique described by Gobbato et al. (7). Briefly, 2 ml of $10^6$ cells/ml extracted from the peritoneum or Peyer's patches were cultured in RPMI-1640 medium (Sigma, St. Louis, USA) and incubated in TC-Plates (6 wells, sterile with LID, Cellstar, Greiner bio-one) for 1 h at 37°C (5% CO2). Plates were washed with RPMI-1640 medium and

adherent cells were infected with 1 ml of $10^7$CFU of *S. Typhimurium*. The bacteria/macrophage ratio was 10: 1. Following a 30 min incubation at 37°C, extracellular bacteria were gently removed by washing with PBS and then RPMI medium containing 100 μg/ml of Gm was added and incubated for 60 min. Finally, the cells were lysed with 1 ml of Triton X-100 1% in RPMI. Serial dilutions of the suspension were plated on MacConkey agar plates. The number of viable intracellular bacteria was measured after incubation for 24 h at 37°C.

*Immunofluorescence assays for detection of IgA, MIP-1α, TLR4 and CD06 positive cells in the lamina propria of the small intestine*

The immunohistochemistry was performed for the adult mice fed with PFM and challenge with *S. Typhimurium*. Small intestines were removed, washed with 0.85% NaCl, cut into pieces and used for histological preparations following the technique described by Sainte-Marie (15). Serial paraffin sections (4 μm) were made and the numbers of IgA positive cells were determined on these histological slides by a direct immunofluorescence assay using an α-chain monospecific antibody conjugated with FITC (Sigma, St Louis, MO, USA).

The cells positive for MIP-1α and those expressing CD-206 and/or TLR-4 were measured on histological slides from the small intestine by indirect immunofluorescence assays using the following primary antibodies: anti murine MIP1-α (Peprotech, USA), mouse anti-human CD-206 (BD Biosciences Pharmingen, USA) monoclonal antibody or rabbit anti-mouse TLR-4 (eBioscience, USA) polyclonal antibody. The sections were then treated with rabbit anti-mouse or goat anti-rabbit antibodies conjugated with FITC (Jackson Immuno Research Labs. Inc., USA).

The number of fluorescent cells was counted in 30 fields of vision at 1000X magnification. Results are expressed as the mean of the number of positive fluorescent cells per 10 fields.

*Statistical analysis*

Statistical analyses were performed using MINITAB 14 software (Minitab, Inc., State College, PA, USA) by ANOVA GLM followed by a Tukey's posthoc test, and $P < 0.05$ was considered significant. All values (N = 15) were the means of 3 independent trials (no significant differences were observed between individual replicates) ± standard deviation.

## RESULTS

*Effect of PFM feeding against S. Typhimurium infection in adult mice. Analysis of pathogen colonization in liver and spleen*

The group of mice fed with PFM before and after salmonella challenge showed significant decreases ($P \leq 0.05$) of the salmonella counts mean log in liver and spleen 7 days post-infection compared to the control group (Fig. 2A). The decreases of salmonella

**Table I.** *Determination of MIP-1α, CD-206 and TLR-4 positive cells in the lamina propria of the small intestine.*

| Sample | Group | MIP-1α | CD-206 | TLR4 |
|---|---|---|---|---|
| Basal | Control | $26\pm7^{a}$ | $13\pm3^{a}$ | $22\pm4^{a,b}$ |
| | PFM | $19\pm8^{a}$ | $14\pm3^{a}$ | $26\pm4^{a}$ |
| 2d p-ch | S | $31\pm9^{a,b}$ | $11\pm2^{a,b}$ | $13\pm4^{c}$ |
| | PFM-S-PFM | $42\pm12^{b}$ | $14\pm3^{a}$ | $14\pm4^{c}$ |
| | S-PFM | $36\pm12^{a,b}$ | $11\pm3^{a,b}$ | $12\pm5^{c}$ |
| 7d p-ch | S | $34\pm6^{a,b}$ | $9\pm1^{b}$ | $18\pm4^{b,c}$ |
| | PFM-S-PFM | $51\pm14^{b}$ | $14\pm3^{a}$ | $13\pm3^{c}$ |
| | S-PFM | $41\pm7^{b}$ | $12\pm3^{a}$ | $14\pm2^{c}$ |

*MIP-1α, CD-206 and TLR-4 positive cells were determined by indirect immunofluorescence on the small intestine tissue slides of mice from different experimental groups in three time points: basal, previous to the infection and 2 and 7 days post-challenge (2d p-ch and 7d p-ch). Results are expressed as number of positive cells counted in 10 fields of vision at 1000X of magnification. Values are means for N = 9 ± SD mice from each group and at each time point. [a,b,c] Means for each cell population without a common letter differ significantly (P < 0.05). PFM = probiotic fermented milk. S = infection control challenged with S. Typimurium. PFM-S-PFM = continuous administration of the PFM before and after challenge. S-PFM = PFM administration after challenge.*

**Table II.** *Total and specific anti-enterobacteria IgA in the fluid of the small intestine.*

| Group | *S. Typhimurium* | |
|---|---|---|
| | Total IgA | Specific-IgA |
| Aa-S-a | 183.13±14.71 | 0.26±0.10$^{a}$ |
| Aa-S-b | 146.69±47.69 | 0.27±0.10$^{a}$ |
| Ab-S-b | 185.61±69.04 | 0.31±0.07$^{a,b}$ |
| Ba-S-a | 159.18±50.35 | 0.44±0.06$^{b}$ |
| Ba-S-b | 160.32±35.29 | 0.24±0.09$^{a}$ |
| Bb-S-b | 188.15±33.00 | 0.19±0.07$^{a}$ |

*ELISA test was used for the determination of total and specific IgA in the fluid of the small intestine of newborn mice from the different experimental groups. The values for total IgA are expressed as concentration (mg/ml), for the specific IgA as OD at 450 nm. Values are means for N = 9 ± SD mice from each group. $^{a,b}$Means for each determination without a common letter differ significantly (P < 0.05).*

**Table III.** *IFNγ and IL-10 in the fluid of the small intestine.*

| Group | *S. Typhimurium* | |
|---|---|---|
| | IFNγ | IL-10 |
| Aa | 365.2±89.5$^{a,b}$ | 291.8±86.5$^{a,d}$ |
| Ab | 292.2±55.3$^{a,c}$ | 345.7±91.8$^{a,b}$ |
| Ba | 424.1±43.6$^{b}$ | 499.6±91.1$^{b,c}$ |
| Bb | 296.9±20.0$^{a}$ | 564.6±43.2$^{c}$ |
| Aa-S-a | 174.9±100.0$^{c,d}$ | 451.9±122.7$^{a,b,c}$ |
| Aa-S-b | 345.9±148.0$^{a,b,c}$ | 464.6±151.1$^{a,b,c}$ |
| Ab-S-b | 269.8±130.1$^{a,b,c}$ | 479.5±52.1$^{b,c}$ |
| Ba-S-a | 329.5±100.2$^{a,b,c}$ | 306.4±88.5$^{a}$ |
| Ba-S-b | 380.9±144.5$^{a,b,c}$ | 288.1±111.4$^{a,d}$ |
| Bb-S-b | 89.5±50.1$^{d}$ | 188.7±55.2$^{d}$ |

*ELISA test was used for the determination of the cytokines in the fluid of the small intestine of newborn mice from the different experimental groups. The values are expressed as concentration (pg/ml). Values are means for N = 9 ± SD mice from each group. $^{a,b,c,d}$Means for each determination without a common letter differ significantly (P < 0.05).*

colonization in liver were also significant in the group of mice fed with PFM only after the infection compared with the controls (Fig. 2A).

*Influence of PFM administration and salmonella challenge on the levels of IgA secreted in the small intestine fluid and on the IgA+ cells associated to the lamina propria of the small intestine of adult mice*

Total IgA concentration increased in the intestinal fluid of mice that only received PFM after pathogen bacteria challenge, 7 days post-infection (322 ± 4 µg / ml), compared to the infection control (274 ±3 9µg / ml) (Fig. 2C). Specific s-IgA against *S. Typhimurium* did not show significant differences (P≤0.05) between the three groups assayed, the mice being administered continuously with PFM before and after Salmonella-challenge, the group with highest O.D. (1.13 ± 0.3) in the intestinal fluid (Fig. 2D).

The determination of the number of IgA producing cells in the small intestine tissues was performed 2 and 7 days post-infection and showed that the pathogen-challenged animals decreased the number of IgA+ cells in the infection control group (58 ± 15 and 77 ± 17, for 2 days and 7 days post-infection, respectively) compared to the basal control, previous to the infection (94 ± 9). The administration of PFM after *S. Typhimurium* challenge increased significantly (P≤0.05) the number of IgA+ cells 7 days post-infection compared with the infection control (Fig. 2E). The group of mice fed continuously with the PFM maintained the number of IgA+ cells similar to the basal data, after 5 days of PFM administration in both 2 and 7 days post-infection samples, with a significant increase compared to the infection control (Fig. 2E ).

*Determination of microbicidal capacity of macrophages isolated from peritoneum and Peyer's patches*

The microbicidal activity against *S. Typhimurium* was assayed in adult mice, in the basal sample (after 5 days PFM administration) using macrophages isolated from peritoneum and Peyer's patches and 2 days post-infection in peritoneal macrophages from the three groups challenged with *S. Typhimurium*. The results showed that 5 days of PFM administration increased the microbicidal activity of macrophages isolated from peritoneum and Peyer's patches, this increase being significant for macrophages from Peyer's patches in comparison with the control without special feeding (Fig. 2B). The microbicidal activity of peritoneal macrophages was increased

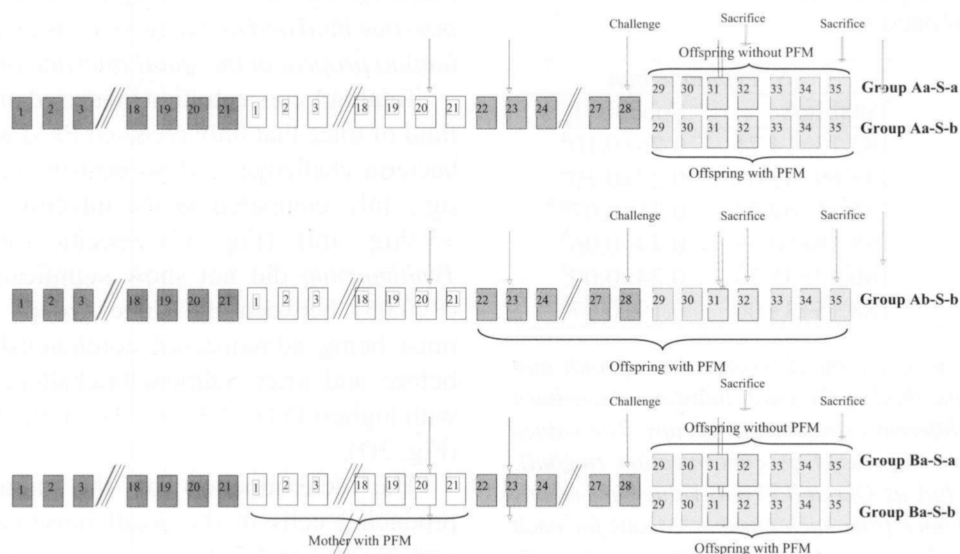


**Fig. 1.** *Design of the different experimental groups of newborn mice under study. Dark gray squares are used for the pregnancy period (21 days approximately); white squares show the suckling period (21 days) and gray squares show the period after weaning, before challenge (28 days of age) and light gray square show the period after S. Typhimuriun challenge until the sacrifice (day 35). Arrows indicate the day of the sacrifice. Brackets are used to indicate the periods when the mothers or their offspring receive or not the PFM. S indicates the pathogen (S. Typhimuriun) challenge.*

2 days post-infection, however no significant differences between the three groups assayed were observed in this sample (Data not shown).

*Influence of PFM administration and S. Typhimurium infection on MIP-1α, CD-206 and TLR-4 positive cells in the lamina propria of the small intestine of adult mice*

The challenge with *S. Typhimurium* induced increases in the MIP-1α+ cell numbers in the lamina propria of the small intestine for 2 days post-infection, compared to the basal data for the control group (25 ± 5) and PFM group (19 ± 8), this increase being significant in the groups of mice fed continuously with PFM before and after pathogen challenge (42 ± 12). For seven days post-infection, the number of MIP1-α+ cells increased significantly in both groups fed with PFM, compared to the basal control, but no significant differences were observed in comparison to the infection control (Table I). The analysis of CD-206+ cells did not show significant differences between the basal data and the three groups challenged with the pathogen, 2 days post-infection (Table I). For seven days post-infection the number of CD-206+ cells decreased significantly in the infection control (9±1) compared to the basal control (13 ± 2).

TLR-4+ cells decreased significantly in the three groups of mice infected with *S. Typhimurium*, compared to the basal data, 2 days post-infection. For seven days post-infection both groups of mice fed with PFM maintained the number of TLR4+ cells significantly low compared to the basal control. No significant differences were observed between the three test groups 2 or 7 days post-infection (Table I).

*Effect of PFM administration against S. Typhimurium infection in offspring whose mothers received PFM during the suckling period or when they were fed with PFM after weaning. Determination of pathogen colonization in liver and large intestine*

The results obtained for liver translocation and large intestine colonization in mice challenged with *S. Typhimurium* showed that the mice whose mothers did not receive PFM during nursing (group A), but

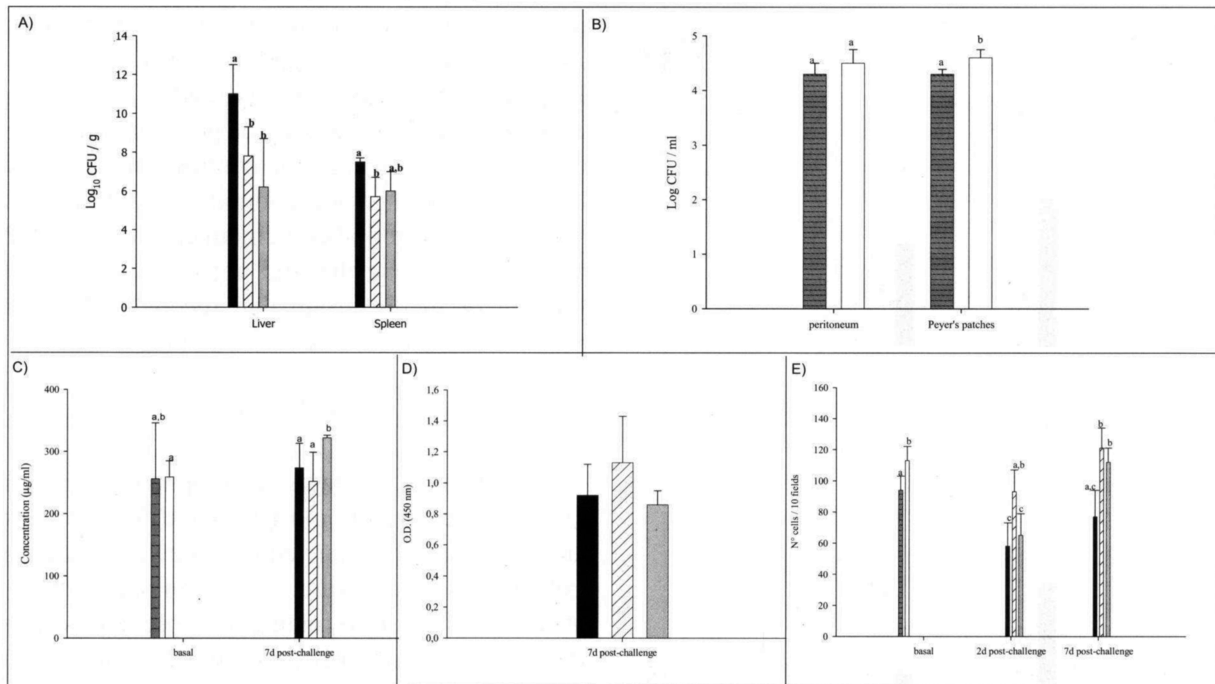


**Fig. 2.** *Effect of PFM administration on S. Typhimurim infection in adult mice.* ***A)*** *The livers and spleens were aseptically removed 7 days post-infection. The samples were homogenized and spread onto the surface of MacConkey agar. Colony counts are expressed as* $log_{10}$ *numbers of bacteria per gram of organ.* ***B)*** *The microbicidal activity of macrophages was determined in the basal sample obtained after 5 days of PFM administration. Results are expressed as* $log_{10}$ *CFU / ml of viable intracellular S. Typhimurium. ELISA was used to measure the concentration of total s-IgA* ***(C)*** *and anti-S. Typhimurium s-IgA* ***(D)*** *in the small intestine fluid obtained from mice of different experimental groups and at different time points: Basal for total IgA and 7 days post-infection for both determinations. Results are expressed for total s-IgA as concentration (mg/ml) and for specific IgA as OD at 450 nm. IgA+ cells* ***(E)*** *were determined by direct immunofluorescence on slides from small intestine of mice and the results are expressed as number of positive cells per 10 fields of vision at 1000X of magnification. The basal sample was obtained after 5 days of PFM administration (white bar) compared to the control without special feeding (gray bar with horizontal lines). The samples post-infection were taken 7 days post challenge in mice from the infection control group (black bars), mice fed continuously (before and after challenge) with PFM (white bars with diagonal lines) and mice fed with PFM after challenge (gray bars). For each assay, values are means for* $N = 9 \pm SD$. $^{a,b,c}$ *Means for each determination without a common letter differ significantly (*$P < 0.05$*).*

were fed with PFM after weaning continuously before and after infection (group Ab-S-b) or as a treatment after the infection (group Aa-S-b), were the mice with absence or significant decreases of salmonella counts mean log in liver and large intestine, compared to the control group which never received special feeding (group Aa-S-a, Fig. 3A). The beneficial effect of the PFM administration to the mothers during nursing was only observed when their offspring did not receive the PFM after weaning (group Ba-S-a). This last group showed significant decreases of salmonella counts in the large intestine, compared to the control (group Aa-S-a, Fig. 3B).

*Analysis of IgA and cytokines secreted in the small intestine fluid*

The analysis of total IgA levels in the small intestine fluid did not show significant differences between any of the groups assayed. Specific anti-*S. Typhimurim* IgA only increased significantly ($P<0.05$) compared to the control (Aa-S-a) in the offspring whose mothers had received PFM during nursing but who did not receive it after weaning (group Ba-S-a). See Table II.

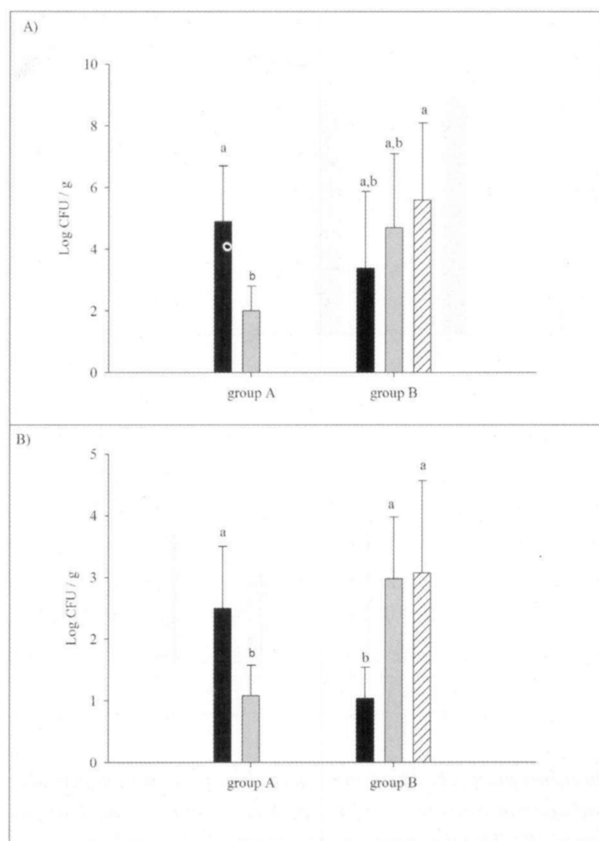


**Fig 3.** *S. Typhimurium colonization in liver and large intestine of newborn mice. The livers and large intestines were aseptically removed 7 days post-infection from mice whose mothers received PFM during suckling period (group B) or mothers who did not (group A). The offspring were divided in three groups: the infection control group (black bars), mice fed after weaning continuously (before and after challenge) with PFM (white bars with diagonal lines) and mice fed with PFM after challenge (gray bars). The livers were spread onto the surface of MacConkey agar (A) and the large intestines were spread onto the surface of Salmonella-Shigella agar. Colony counts are expressed as $log_{10}$ numbers of bacteria per gram of organ. Each point represents the mean of $N = 9 \pm SD$. [a,b]Means for each organ without a common letter differ significantly ($P < 0.05$).*

The study of the cytokines was performed in two time points: the basal data, at day 28, the same day of the infection, and 7 days post-infection. Basal data showed significant increases for IL-10 concentration in the intestinal fluid from mice whose mothers received PFM during nursing. No significant differences were observed for IFNγ concentration at this time point (Table III). After infection, mice from mothers that had not received PFM showed a high level of IL-10, and the concentrations of IFNγ were maintained increased when the offspring received PFM continuously or post-infection, these levels being higher than the control (group Aa-S-a). Mice whose mothers were administered PFM during the suckling period also maintained increased IFNγ levels in the group that did not receive PFM after weaning or in the group that received PFM after infection, compared to the control (Table III)

## DISCUSSION

The prevention against enterophagenic bacteria is one of the benefits most reported for probiotics, but little research is carried out to evaluate whether probiotic administration is required after an intestinal infection to maintain its preventive effect or if treatment with only probiotic after the infection could be useful.

The results obtained in the present work show that the administration of PFM to adult mice, continuously before and after *S. Typhimurium* challenge or as a treatment after the infection, decreased the mortality (data not shown) and the spread of the pathogen to organs, diminishing the severity of the infection. PFM administration to adult mice increased the number of IgA+ cells in the lamina propria of the small intestine of mice challenged with *S. Typhimurium*; this observation was important because IgA+ cells are involved in one of the first steps of the mucosal immune stimulation (16) and it is know that s-IgA antibodies are major effector molecules in the mucosal system (17-18). However, total IgA was only increased in the intestinal fluid of mice fed with PFM after infection.

Considering that PFM administration to adult mice increased the number of macrophages and dendritic cells and the phagocytic activity of peritoneal macrophages in mice fed long- term with it (11), the chemokine MIP1-α and the microbicidal activity of macrophages were studied in the model of salmonella infection. The salmonella challenge increased the number of MIP-1α+ cells in the small intestine of the mice 2 days post-infection, showing the importance of the cell attraction to the intestine in the response against the pathogen. In

this sense, MIP1-α can be produced by monocyte/macrophages in response to the LPS stimulus and act as a chemoattractant to a variety of cells (19). It was observed that the continuous PFM administration was important to have a fast reaction of the immune response with increases of MIP-1α+ cells from 2 days post-challenge. Considering that the increases of the chemokine MIP-1α are related with an increase in cells of the first line of the immune response such as macrophages, the microbicidal capacity of these phagocytes was evaluated in Peyer's patches. This activity was significantly increased the day of the infection in mice that received PFM. This observation again demonstrates the importance of the continuous probiotic consumption for an optimal intestinal immune surveillance.

The study of possible cell receptors involved in the immune activation induced by *S. Typhimurium* and/or by the PFM, showed that PFM administration increased CD206 + cells in both groups studied, 7 days post-infection. This observation agrees with other probiotics that increased this receptor in the immune cells of the innate immune response in both lamina propria and Peyer's patches of the small intestine (20). This mannose receptor is mainly implicated in the homeostatic system for the clearance of endogenous molecules, although its participation in the context of the innate immune response needs to be clarified (21). Another receptor analyzed was TLR-4 because it recognizes the LPS present in the membrane of the Gram(-) bacteria, such as salmonella (22). It is known that TLR-4 is required to control salmonella infection. The activation of this receptor initiates an innate immune response leading to the induction of proinflammatory mediators but then leads to the suppression of its own mRNA expression during salmonella infection (23), which agrees with the results obtained in our study where, after *S. Typhimurium* infection the number of TLR-4+ cells decreased in all the groups compared with the basal data. These decreases were more important for the mice fed with PFM continuously or after salmonella challenge, which could be related with the decrease in the severity of the infection for these groups where the bacterial growth in spleen and liver decreased faster than in the infection control. Another important consideration is the fact that in the basal sample, before the infection, PFM maintained increased the number of TLR-4+ cells, which, as explained above, is very important in the fight against salmonella challenge.

In the second part of the study, infections to newborn mice were considered. PFM administration to the newborn mice after weaning showed the best effect against *S. Typhimurium* when their mother did not received the PFM. PFM administration to the mother during the suckling period could be beneficial, especially when their offspring do not receive probiotics after weaning. Mice fed with PFM after weaning and from mothers that received PFM during nursing showed a regulated immune maturity that did not protect against salmonella infection. The increased levels of IL-10 at the time of infection were related with a suppressor response that the pathogen used to draw a normal inflammatory response. This observation can be related with the previous report where it was shown that the administration of this PFM to the mothers during the suckling period improved their own immune system and this was reflected in their offspring, conferring them a more regulated maturation of the immune cells involved in the innate immunity and on IgA+ cells. It is possible that this regulation could be useful against other pathogens or inflammatory stimuli, and to know more about the importance or not of this immune regulation other experimental models are currently under study.

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