EFFECT OF YOGHURT ON THE CYTOKINE PROFILE USING A MURINE MODEL OF INTESTINAL INFLAMMATION

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Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, are important problems in industrialized countries. The complete aetiology of both diseases is still unknown but likely involves genetic, environmental and immunological factors. The aim of this work is to study the anti-inflammatory mechanisms reported for yoghurt in a colon cancer model in order to evaluate its usefulness in the treatment of intestinal inflammation such as Crohn's disease. A trinitrobenzenesulfonic acid (TNBS)-induced colitis model was used. The influence of yoghurt feeding was studied before and after TNBS inoculation. The effect on the intestinal microbiota and on the host immune response was evaluated. IgA-producing cells and cells positive for specific cytokines (IL-12, IL-17, IFNγ and IL-10) were analyzed. Yoghurt administration diminished the severity of inflammation in the TNBS-inoculated mice. This effect occurred mainly through IL-10, which was increased in the intestinal tissues throughout the study, and by the decrease observed in IL-17 and IL-12 levels. In addition to this immunomodulatory capacity, another mechanism by which yoghurt could exert the anti-inflammatory activity observed in our model would be through beneficial changes in the intestinal microbiota (increases in the bifidobacteria and lactobacilli populations). These changes in the intestinal microbiota could also be considered one of the causes of the intestinal inflammation reduction. These results show that yoghurt administration modulated the immune response, inducing down regulation of the inflammatory cytokines produced by the immune cells involved in the inflammatory process. The protective effect of yoghurt could also be mediated through beneficial changes in the intestinal microbiota favouring lactobacilli and bifidobacteria population.

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, are important problems in industrialized countries and have been associated with increased colon cancer risk (1). The complete aetiology of both diseases is still unknown but it is likely that it involves genetic, environmental and immunological factors. IBD is a chronic immune-mediated disease in which endogenous bacteria are thought to play an important role, as suggested by numerous clinical observations and experimental studies. There is a growing body of evidence that supports the role of intestinal microbiota in the initiation and progression of IBD (2).

Experimental models of colitis are used because they imitate human IBD, ulcerative colitis, Crohn's disease and chronic relapsing diseases of unknown aetiology (3). These models showed that probiotics can confer beneficial effects on

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intestinal inflammation partially because of their immunomodulatory effects. Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the consumer (4). Probiotics can be useful in the prevention or treatment of IBD because they can improve the intestinal microbial balance and the intestine immunologic barrier, particularly through immunoglobulin (Ig) A response and alleviation of intestinal inflammatory responses, which produce gut-stabilizing effects (5).

Lactic acid bacteria (LAB) are present in many foods, including yoghurt, and are frequently used as probiotics to favour biological functions in the host. Yoghurt has been defined in the Codex Alimentarius (2002) as a coagulated milk product that results from the lactic acid fermentation of milk by *Lactobacillus* (*L.*) *delbrueckii* subsp. *bulgaricus* and *Streptococcus* (*S.*) *thermophilus*. In this definition, in addition to the yoghurt starter cultures, the peptides and other substances released during the fermentation process can also be important. Many researchers have studied the therapeutic effects of yogurt and LAB commonly used in yogurt production against diseases such as cancer, infection, and gastrointestinal disorders. The immunomodulating and immunostimulating properties of yogurt have also been well documented (6). The regulation of the inflammatory immune response is one of the mechanisms by which yoghurt could prevent the risk of colon cancer in an experimental model, and could be effective in models of intestinal inflammatory disease.

Modification of the Th1 and Th2 subpopulations in favour of the Th1 population seems to be one of the mechanisms that can produce the development of chronic intestinal inflammatory diseases. This concept is reinforced by different studies that demonstrated increases in pro-inflammatory cytokines produced by this subpopulation. In this way, the suppression or regulation of Th1 response can be an alternative for the treatment of IBD in animals and humans. Numerous works have shown the importance of regulating IL-10 cytokine in the modulation of the immune response at intestinal level, and mice deficient in this gene that encodes this cytokine have been used as models of intestinal inflammation (7).

The aim of the present work is to study the anti-inflammatory mechanisms previously reported for yoghurt in a colon cancer model to evaluate whether this product could prevent or be useful for the treatment of intestinal inflammation. In order to meet this objective, a TNBS induced colitis model was used and the influence of yoghurt feeding on the intestinal microbiota and on the anti-inflammatory gut immune response was evaluated.

MATERIALS AND METHODS

Experimental groups

Five-week-old female BALB/c mice weighing 25-28 g were obtained from the random-bred colony maintained at CERELA and divided into five experimental groups. 1) TNBS group: The mice received inoculation of trinitrobenzenesulfonic acid (TNBS) to induce inflammation; 2) yoghurt-TNBS-yoghurt group: The mice were fed with yogurt for ten consecutive days (basal yogurt), treated with TNBS, and then fed again with yogurt; 3) yoghurt-TNBS group: The mice were given yogurt for 10 consecutive days and then inoculated with TNBS to study the effect of yoghurt on the prevention of the inflammation; 4) Control group: The mice were inoculated with the TNBS vehicle (phosphate buffered saline, PBS in ethanol 50% without TNBS); 5) yogurt control group: Mice were fed with yogurt during all the experiment but without TNBS inoculation.

All groups were fed *ad libitum* with a balanced diet and were maintained in a room with a 12-h light/dark cycle at 18±2ºC. Each experimental group consisted of 20-25 mice.

All animal protocols were preapproved by the Animal Protection Committee of CERELA and all experiments complied with the current laws of Argentina.

Yoghurt preparation

Simulated commercial yogurt was freshly prepared and controlled every day to keep the number of bacteria constant and to avoid variations due to storage. Yoghurt was prepared from cultures of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (strain pools were used from the CERELA culture collection). The total number of bacteria in the fermented product was 2×10^9 CFU/ml.

Induction of intestinal inflammation and feeding procedure

For induction of the intestinal inflammation, mice (which were anesthetized intraperitoneally using a mix of ketamine hydrocholoride (Hollyday-Scott S.A., Buenos Aires, Argentina), 100 µg/g body weight, and xylazine hydrochloride (Rompun, BAYER, Division Sanidad

Animal, Buenos Aires, Argentina), 5 µg/g body weight) received by intrarectal administration 100 µl of a solution of TNBS (Sigma, St Louise; 2mg/mouse) dissolved in PBS 0.01 M, pH 7.4 and mixed with an equal volume of ethanol (50% ethanol). Control mice received PBS mixed with ethanol (without TNBS) using the same technique.

Body weight of mice was controlled every day until the end of the experiment.

Sampling procedures and colon histology

The following samples were obtained from each group: basal sample (day 0, the same day of TNBS inoculation and previous to it), and 3, 7 or 14 days after TNBS inoculation. Ten mice for each group were sacrificed at each sampling period. Five mice were sacrificed for histological and immunohistochemical evaluations. The experiments were repeated 3 times. Serial paraffin sections of 4 µm were made and stained with hematoxilin-eosin for light microscopy examination. For the colon histology, the microscopic slides were reviewed and the extent of colonic damage and inflammation was assessed using the histopathological grading system of Ameho et al (8) (Table I). The other five mice (N=5) of each group were analyzed for the liver translocation and to study the microbiota in the large intestine.

Study of the intestinal microbiota

The large intestine of mice 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 homogenizer (MSE, England). Serial dilutions of the homogenized samples were obtained and aliquots (0.1 ml) of the appropriate dilution were spread onto the surface of the following agarized media: Reinforced Clostridial agar (RCA, Britania, Buenos Aires, Argentina) for total anaerobic bacteria; RCA containing 0.2% LiCl, colistin 4mg/L, 1% aniline blue and after sterilization adjusted to pH 5 with acetic acid (RCA-pH5) for isolation of bifidobacteria; Mann-Rogosa-Sharp Agar (MRS Britania, Buenos Aires, Argentina) for total lactobacilli and MacConkey (Britania, Buenos Aires, Argentina) for Enterobacteriaceae. This last culture media was aerobically incubated at 37ºC for 24 h, all others plates were anaerobically incubated at 37ºC for 72-96 h.

Determination of bacterial translocation to liver

Livers were aseptically removed, weighed and placed into sterile tubes containing 5 ml of peptone water and mechanically homogenized under sterile conditions using a homogenizer (MSE, England). The samples were homogenized, and cultures were performed on Mann-Rogosa-Sharp Agar, MacConkey agar and LAPTg (yeast extract, peptone, tryptone and glucose). The cultures were aerobically incubated at 37°C for 24-72 h.

Immunofluorescence assay for IgA-secreting cells in the large intestine

Large intestine tissue sections (4 µm) from each mouse were used for the immunofluorescence assays. The numbers of IgA(+) cells were determined by direct immunofluorescence assay. Slides were incubated with α-chain monospecific antibody conjugated with fluorescein isothiocyanate (FITC, Sigma, St Louis, USA). The number of fluorescent cells was counted in 30 fields of vision, as seen at 1,000x magnification using a fluorescent light microscope. The results were expressed as positive cells in ten fields of vision.

Cytokine-producing cell determination in histological sections

Cytokine positive cells were detected by indirect immunofluorescence on large intestine tissues, following the technique described by de Moreno de LeBlanc and Perdigon (6). Rabbit anti-mouse IFNγ and IL-10, or goat anti-mouse IL-12 polyclonal antibodies (Peprotech, Inc. Rocky Hill, NJ, USA) and goat anti-mouse IL-17 polyclonal antibody (R&D System, Minneapolis, USA) (diluted in saponin-PBS) were applied to the sections for 75 min at room temperature (21ºC). The sections were then treated with a dilution of goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC, Jackson Immuno Research, Labs. Inc., West Grove, USA). The number of fluorescent cells was counted in thirty fields of vision as seen at 1,000x magnification using a fluorescence light microscope and expressed as number of positive cells in ten fields of vision.

Statistical analysis

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

RESULTS

Effect of yoghurt in TNBS-treated mice. Analysis of mortality, body weight and intestinal histology

Mice treated with TNBS (2mg/mouse) showed 25-30% mortality rate which was associated with a significant loss of body weight (Fig. 1) and high

Table I. *Histopathological grading scale of chemically induced colitis.*

Grade	Microscopic findings
0	Histological findings identical to normal mice
1	Mild mucosal and/or submucosal inflammatory infiltrate (admixture of neutrophils) and oedema. Punctate mucosal erosions often associated with capillary proliferation. Muscularis mucosae intact
2	Grade 1 changes involving 50% of the specimen.
3	Prominent inflammatory infiltrate and oedema (neutrophils usually predominating) frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa. Rare inflammatory cells invading the muscularis propriae but without muscle necrosis
4	Grade 3 changes involving 50% of the specimen
5	Extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells. Necrosis extends deeply into the muscularis propria
6	Grade 5 changes involving 50% of the specimen

Table II. *Colon microscopic scores of mice.*

Group	Days post-TNBS		
	3	7	13
Control	0.8 ± 0.7^{a}	0.6 ± 0.4^{a}	0
Y-TNBS	$1.6\pm1^{a,b}$	$2.9\pm0.9^{b,c}$	$1.5\pm1^{a,b}$
Y-TNBS-Y	$1.4\pm0.9^{a,b}$	2.5 ± 0.6^{b}	$1.2\pm0.7^{a,b}$
TNBS	2.3 ± 0.7^{b}	4.7 ± 1.2^{c}	3.3 ± 0.6^{c}

Each value represents the mean of N = 15 ± SD. Means for each value without a common letter differ significantly ($p < 0.05$). Y = yoghurt

levels of colitis (intestinal histological alterations). The TNBS control group showed the highest weight loss that became significant 3 days post inoculation (27.4 ± 1.5 and 22.5 ± 1.4 for 1 and 3 days, respectively); severe mucosal damage that included a loss of crypts, necrosis, and focal influx of inflammatory cells in the mucosa and submucosa were observed (Fig. 2E). The higher score of colonic damage was obtained 7 days post-TNBS (4.7±1.2, Table II). Decrease in the goblet cell counts was another characteristic of this group. Premature death resulted mainly from an excessive inflammatory reaction, as assessed by post-mortem autopsy and considering the histology and other parameters studied in the mice sacrificed during the study, this group should have a greater mortality rate.

The greater number of mice that received exclusively the vehicle had similar macroscopic and microscopic appearance of their colons compared to the normal mice (Fig. 2A). A small number of these animals had mucosal or submucosal infiltrates focalized in regions of the colon.

The administration of yoghurt increased mononuclear cell numbers in the lamina propria of

Table III. *Bacterial translocation to the liver.*

Group	Sample	MacConkey	LAPTg	MRS
Control	Basal	WG	2.4±1.0 [a]	WG
	3d post TNBS	2.1±0.9 [a,b]	3.8±1.3 [a]	2.4±0.8 [a,b]
	7d post TNBS	WG	0.9±0.7 [a]	1.6±0.1 [a]
	13d post TNBS	WG	WG	WG
Yoghurt	Basal	WG	2.5±1.5 [a]	WG
	3d post TNBS	1.7±0.3 [a]	2.3±1.8 [a]	1.7±0.1 [a]
	7d post TNBS	1.6±0.9 [a,b]	1.9±1 [a,b]	1.6±0.2 [a]
	13d post TNBS	WG	1.5±0.9 [a]	WG
Y-TNBS	Basal	WG	2.5±1.5 [a]	WG
	3d post TNBS	3.9±1.6 [b,c]	4.9±1.1 [a]	2.4±1.2 [a,b]
	7d post TNBS	WG	1.4±0.6 [a]	2.9±2.0 [a]
	13d post TNBS	WG	WG	3±1.6 [a]
Y-TNBS-Y	Basal	WG	2.5±1.5 [a]	WG
	3d post TNBS	3.0±1.0 [a,b]	4.0±1.5 [a]	2.2±0.6 [a,b]
	7d post TNBS	1.3±0.7 [a]	1.7±0.7 [a]	1.5±0.1 [a]
	13d post TNBS	WG	WG	2.7±1.0 [a]
TNBS	Basal	WG	2.4±1.0 [a]	WG
	3d post TNBS	5.2±0.5 [c]	3.6±0.9 [a]	3.9±2.0 [b]
	7d post TNBS	2.9±0.8 [b]	4.0±1.4 [b]	5.1±1.4 [b]
	13d post TNBS	WG	0.3±0.2 [b]	2.9±0.2 [a]

Colony counts are expressed as log_{10} numbers of bacteria per gram of liver. Each value represents the mean of N = 15 ± SD. For each time point, comparing all the groups, means for each culture medium without a common letter differ significantly ($p < 0.05$).
WG = without growth in the sample without dilution, Y = yoghurt

the large intestine as was reported previously by de Moreno de LeBlanc (9) but these were focalized near other histological unaltered areas (Fig. 2B).

Ten days of yoghurt feeding previous to the TNBS inoculation did not significantly decrease the mortality rate, but the body weight did not decrease significantly compared with the basal data. Mice from this group showed an important reduction of the colonic damage caused by the inflammatory agent (Table II). Although some infiltrates in mucosa and submucosa were present, most of the tissues of the large intestine were similar to those observed in the alcohol control animals (Fig. 2C).

The group of mice that received continuous administration of yoghurt (before and after TNBS) showed decreased mortality rates compared to the inflammation control group, however these animals did not significantly lose body weight ,and the histological observations showed similar characteristics to the last described group with an important increase in the number of goblet cells (Fig. 2D and Table II).

Changes in the intestinal microbiota by yoghurt administration and bacterial translocation to liver

Mice fed 10 days with yoghurt showed a significant increase in the bifidobacteria counts (mean log CFU of 6.5 ± 1.0) compared to the control group that did not receive fermented milk (3.1 ± 0.2, Fig. 3A).

Another bacterial population that increased significantly in mice receiving yoghurt was total lactobacilli (8.0 ± 0.3 and 7 ± 0.1 for yoghurt and control group, respectively; (Fig. 3A).

These increases (bifidobacteria and lactobacilli) were maintained in mice that received yoghurt after TNBS inoculation but not in mice that stopped yoghurt administration (yoghurt-TNBS group, Fig. 3B). Significant differences between groups were not observed for the other culture media assayed

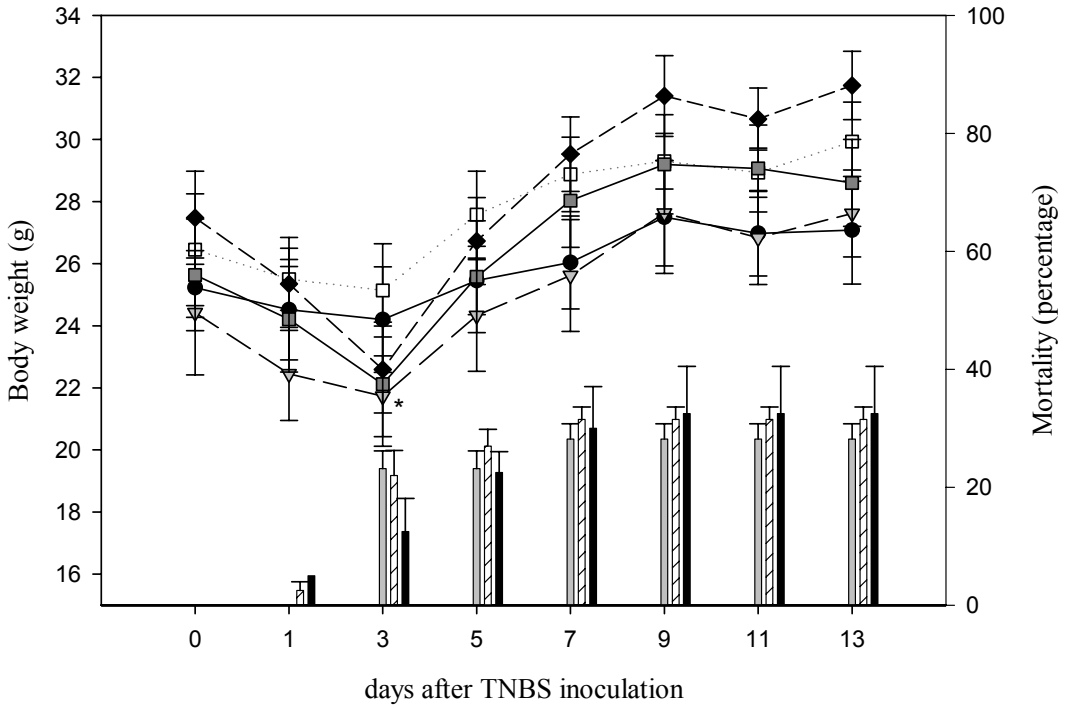

Fig. 1. *Changes in the body weight, mortality percentage and histological parameters.*
*Weight changes of normal control mice treated with 50% ethanol (black whole line and circle) and the test groups: yoghurt control (black dotted line and empty square); yoghurt-TNBS (black long dash line and gray triangle); yoghurt-TNBS-yoghurt (black whole line and gray square); TNBS (black long dash line and diamond). Weight data from all experiments are shown. Each point represents average ± SD of weight data pooled from 45 mice (15 mice from each separate trial). * significant difference ($p < 0.05$) is observed only for TNBS group 3 days after TNBS inoculation, compared with the basal data for the same group. Mortality was only observed for the groups inoculated with TNBS: yoghurt-TNBS (gray bars); yoghurt-TNBS-yoghurt (diagonally lined bars) and TNBS (black bars). Results are expressed as means ± SD of the mortality of 45 mice (15 mice in each three different experimental trials) for each group during the period of the experiment.*

(data not shown).

Bacterial translocation in the liver showed that mice treated with TNBS (TNBS group) increased the number of CFU in the three growth media assayed (MacConkey, LAPTg and MRS). This increase was higher on days 3 and 7 post-induction of the inflammation and decreased afterwards, when the mice that survived improved their inflammatory condition (Table III). Mice from the group that received yoghurt 10 days before TNBS inoculation showed lower translocation than the TNBS group, and values returned to basal numbers of CFU in liver at day 7 post-TNBS. Similar results to this last group were obtained in mice that received continuous yoghurt administration (before and after TNBS). No significant differences were observed when these results were compared to the yoghurt control group.

Study of IgA+ cells in the large intestine

Yoghurt feeding increased the number of IgA+ cells in the large intestine of mice; this was observed in the yoghurt control group in the second and third samples. When mice were treated with TNBS, yoghurt administration increased the number of IgA+ cells compared to the TNBS control group. When the fermented milk was administered continuously; this effect was significant in the last sample (74 ± 10 for yoghurt-TNBS-yoghurt group and 57 ± 6 for TNBS group, Fig. 4).

Study of cytokine positive cells in the large intestines

TNBS-induced inflammation increased the

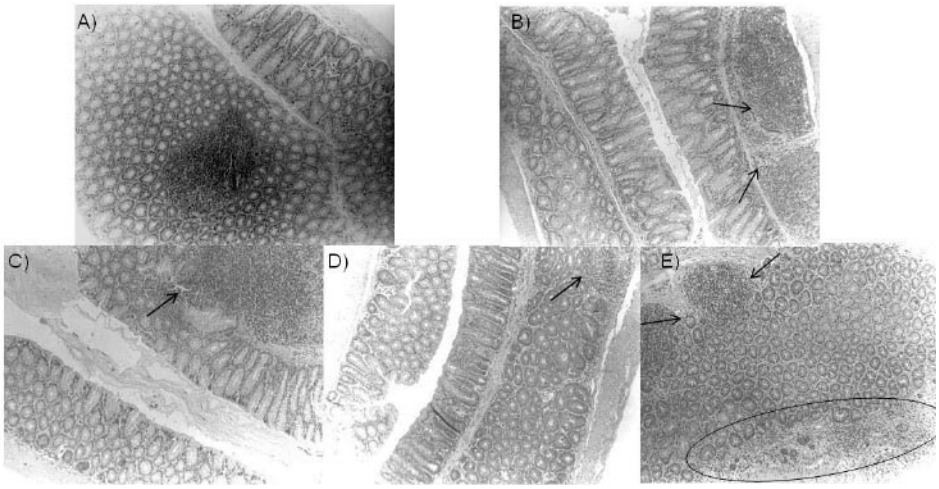

Fig. 2. *Histological study comparing the different experimental groups. Slices from large intestine of mice were studied after staining with hematoxilin-eosin. All the samples were obtained 7 days post-TNBS with a magnification of 100x.* ***A)*** *Alcohol control group.* ***B)*** *Yoghurt group. Tissue maintained the typical structure of the large intestine but it is possible to observe immune cell infiltration in specific areas (arrows).* ***C)*** *and* ***D)*** *Yoghurt-TNBS and yoghurt TNB-yoghurt group: In both groups there are cells infiltrating mucosa and submucosa (arrows) but most of the tissues of the large intestine were similar to those observed in the alcohol control animals. E) TNBS group: Severe mucosal damage is observed with loss of crypts (encircled), focal influx of inflammatory cells in the mucosa and submucosa (arrows). Decrease in the goblet cell was another characteristic of this group.*

number of IFNγ and IL-12 positive cells in the two first samples (29 ± 8 and 24 ± 3 for IFNγ, and 25 ±3 and 20 ± 2 for IL-12, 3 and 7 days post TNBS, respectively (Fig. 5). Yoghurt feeding decreased the number of positive cells for both cytokines, IFNγ in the two same time points and IL-12 in the second sample taken 7 days post-TNBS (16 ± 5 and 17 ± 2 for previous and continuous yoghurt feeding), showing a regulation of the inflammatory immune response. For IL-12+ cells, mice given yoghurt (yoghurt control group) did not show significant differences compared to the control group without inflammation (alcohol group). IL-17 was another cytokine studied because it has been reported in several inflammatory pathologies. In our model, IL-17+ cells increased in all the groups treated with TNBS, 3 days after the inoculation (32 ± 6, 23 ± 10 and 22 ± 8 for TNBS, yoghurt-TNBS and yoghurt-TNBS-yoghurt groups, respectively) compared with the control group without inflammation (12 ± 1). In the other two samples taken 7 and 13 days post-TNBS, the increased number of these cells was maintained for the TNBS group, but not for the mice given yoghurt (Fig. 5).

Yoghurt feeding by itself did not increase IL-12 or IL-17 secreting cells, compared to the control without inflammation, but in the assay for IFNγ+ cells it was observed that yoghurt administration significantly increased the number of the cells positive for this cytokine in the basal sample and 3 days post-TNBS.

IL-10 was another cytokine studied and it was observed that mice fed with yoghurt increased numbers of IL-10+ cells compared to the TNBS control (Fig. 5). These increases were higher in mice that received continuous yoghurt administration, including after TNBS inoculation.

DISCUSSION

Previous works showed that yoghurt can prevent the growth of a chemically induced colon cancer in mice. This effect was attributed to its anti-inflammatory activity. Yoghurt can modulate the immune response by 1) stimulating cytokine production when this is required, or 2) inducing down-regulation of the immune cells to avoid an exacerbated immune response. This effect would

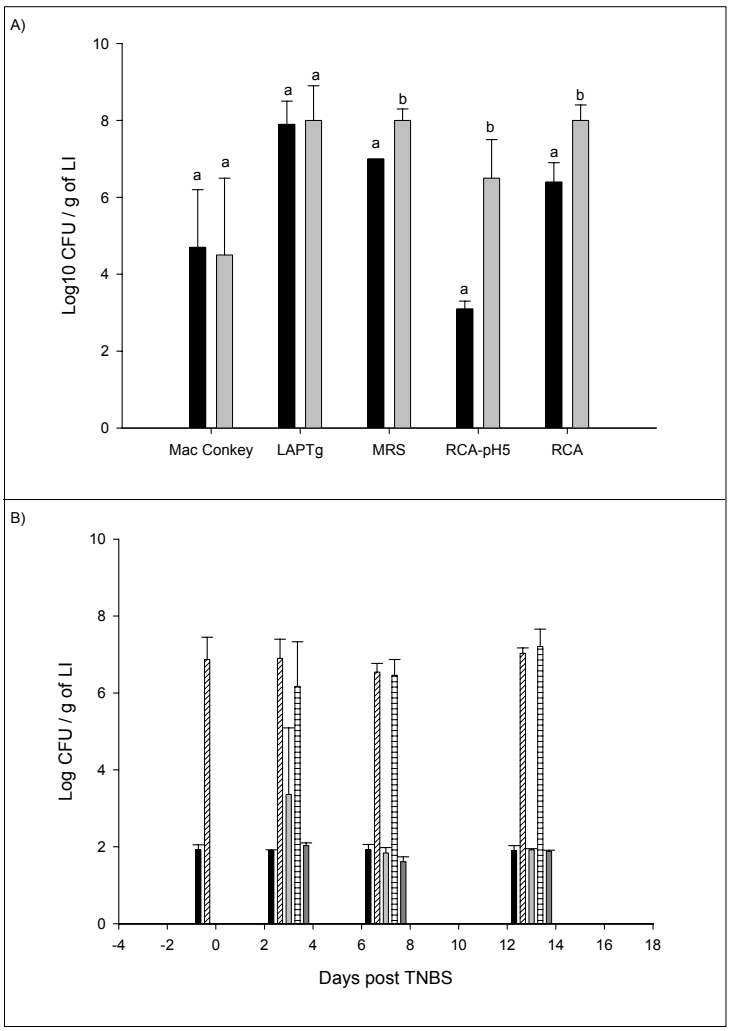

Fig. 3. *Effect of yoghurt feeding and TNBS inoculation on the microbiota of the large intestine. Results are expressed as means ± SD of the log_{10} CFU/g large intestine (LI).* ***A)*** *Represents the basal data, previous to the TNBS inoculation, for control mice (black bars) and mice fed 10 days with yoghurt (gray bars).* ***B)*** *Represents the results obtained for bifidobacteria (RCA-pH5 medium) in all the groups assayed. Mice from yoghurt-TNBS group showed growth in this culture medium only in the first sample (3 days post TNBS). Mice fed continuously with yoghurt [yoghurt (diagonal bars) and yoghurt-TNBS-yoghurt (horizontal bars) groups] showed growth in all the samples assayed. For the other groups (50% ethanol control and TNBS) no colonies were present in the lowest dilution assayed (10^{-2}). Each mean represents data from five animals. $^{a–b}$Means for each medium without a common letter differ significantly ($p < 0.05$).*

occur mainly through IL-10, which was increased in the tissue of the mice receiving yoghurt (10).

In the present study, the effect of yoghurt was evaluated using a murine model of intestinal inflammation. The inflammation induced by TNBS was selected as a model because it shares important similarities with human Crohn's disease such as transmural inflammation, lymphocyte infiltration and Th1-dominated cytokine profiles (11-12).

In the present work it was observed that yoghurt feeding previous to the TNBS inoculation or continuously (before and after TNBS), reduced the colonic damage caused by the inflammatory agent (Table II); the group of mice that received continuous administration of yoghurt showed decreased mortality rates compared to the inflammation control (Fig. 1).

These results led us to study the possible mechanisms by which yoghurt feeding can exert its beneficial effects on the TNBS-treated mice.

Most of the models in which animals develop spontaneous or chemically-induced colitis are influenced by the microbiota present in the intestinal lumen. This fact is supported by the reduction or absence of intestinal inflammation in TNBS or dextran sulphate sodium (DSS) models of colitis using antibiotic-treated and germ-free animals (13-14). In addition, many studies have shown

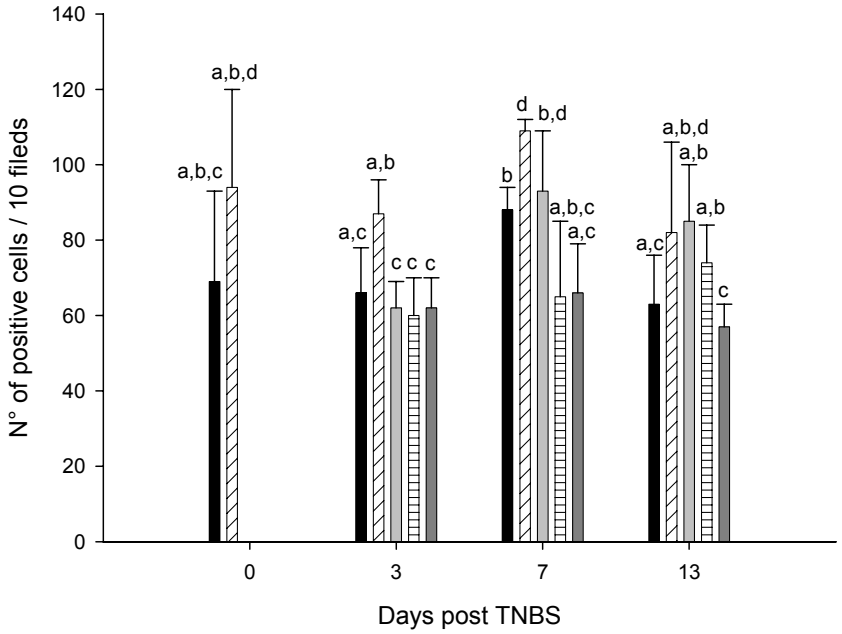

Fig. 4. *Effect of TNBS-induced inflammation and yoghurt feeding on IgA+ cells in the large intestine. Positive cells were counted in histological sections from large intestine of alcohol control (black bars), yoghurt (diagonally lined bars), yoghurt-TNBS (light gray bars), yoghurt-TNBS-yoghurt (horizontal lined bars) and TNBS (dark gray bars) groups. Data correspond to the means ± SD of results of 15 animals from three separate experiments.* ^{a,b,c,d}*Means for each value without a common letter differ significantly ($p < 0.05$).*

that not all bacterial species have equal activities in promoting or reducing intestinal inflammation.

In the present study, when the basal sample, taken after 10 days of yoghurt feeding (the day of TNBS inoculation), was compared to mice that did not receive yoghurt previously, changes in the intestinal microbiota were observed. Yoghurt feeding increased the bifidobacteria and lactobacilli counts. The relationship between the administration of fermented milks containing lactobacilli and the increase in *Bifidobacterium* counts has been previously reported (15). This fact can be related with recent findings that some LAB strains have metabolic pathways needed for the synthesis and release of molecules that selectively stimulate the growth of endogenous bifidobacteria. It has been suggested that the increase of these bacteria could confer a beneficial effect on the stability of the intestinal microbiota (16). The increase in lactobacilli count was in agreement with other publications where the intake of probiotic bacteria or fermented milk caused the same effects (17).

Bifidobacteria and lactobacilli are known to be desirable microbiota inhabitants of the intestines and are related with many beneficial effects in the gastrointestinal tract such as the prevention of inflammation.

The TNBS model is associated with the absence of LAB and an increase in other aerobic isolates such as *Escherichia coli* and *Staphylococcus* spp. (18). Similarly, decreased levels of faecal lactobacilli and bifidobacteria have also been reported in Crohn's disease (19).

The mechanisms whereby probiotics exert their effects in general, and in IBD in particular, are not yet fully understood. Some LAB and bifidobacteria can produce and release specific low molecular weight anti-microbial substances (bacteriocins) (20). Some probiotic bacteria are capable of normalizing increased intestinal permeability to potentially antigenic macromolecules (21); another characteristic of intestinal inflammation is the increase in the permeability of the colonic mucosa barrier and a decrease in IgA levels. All these parameters contribute to bacterial translocation to

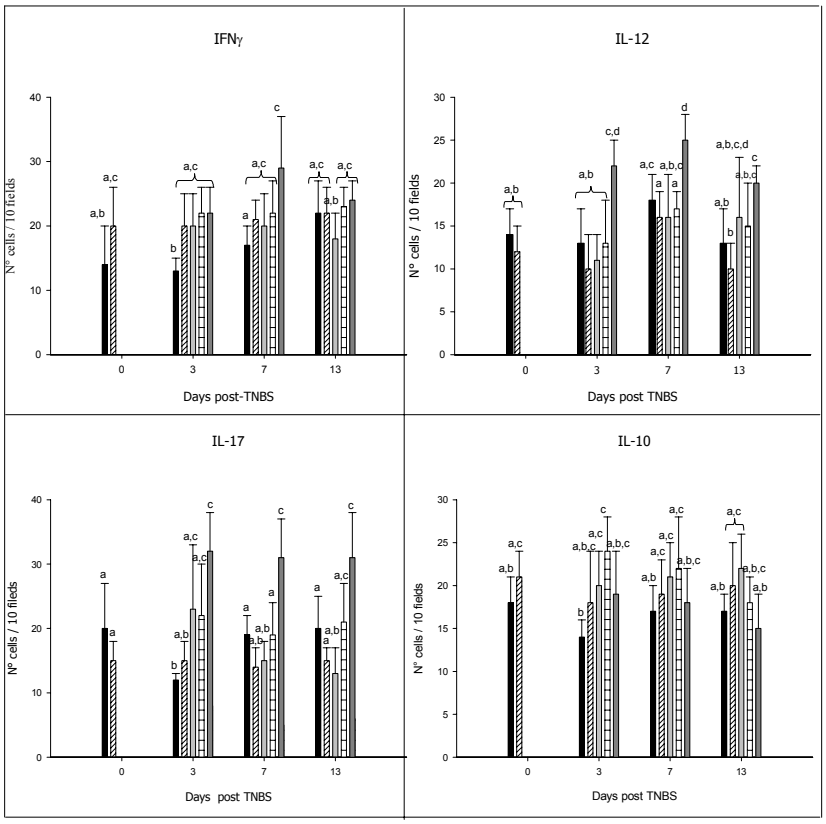

Fig. 5. *Effect of TNBS inoculation and yoghurt feeding on the cytokine+ cells of the large intestine. Positive cells for each cytokine were counted in histological sections from large intestine of alcohol control group (black bars), yoghurt (diagonally lined bars), yoghurt-TNBS (light gray bars), yoghurt-TNBS-yoghurt (horizontal lined bars) and TNBS (dark gray bars) groups. Data correspond to the means ± SD of results from 15 animals of three independent experiments.* ^{a,b,c,d}*Means for each cytokine without a common letter differ significantly ($P < 0.05$).*

other organs.

In this study, the lesser extent of colonic damage in the intestine from mice which received yoghurt was related with a decrease in the bacterial translocation to the liver, compared to the mice from the TNBS control group (Table III). All these observations confirm that yoghurt feeding diminished the severity of inflammation in the mice and that changes in the intestinal microbiota at the colitis induction site could be a contributing factor to this beneficial effect.

Considering previous results where yoghurt feeding showed anti-inflammatory properties by modulating the immune response (22), the study of its effect on the intestinal immunity was evaluated.

Probiotics can modify the immune functions of the host, although the specific mechanisms remain unclear. Enhanced intestinal IgA production, which provides defence for the mucosal surface (23), has been reported as one of the beneficial effects associated to probiotic consumption.

In the present study, yoghurt feeding increased the number of IgA+ cells in the large intestine of mice; this was observed in the yoghurt control group and also in mice treated with TNBS, in which yoghurt administration increased the number of IgA+ cells compared to the TNBS control group (Fig. 4).

These results agree with the increase of IgA reported in connection with the administration of *L. rhamnosus* GG in Crohn's disease (24) and with our previous results where the administration of yoghurt

increased the number of IgA+ cells in a colon cancer model in mice. The increase in this cell population but not in IgG secreting cells in the large intestine of mice fed with yoghurt could limit the inflammatory response, since IgA is considered an important barrier in colonic neoplasia (10).

An effective inflammatory immune response initially requires recruitment of immunocompetent cells to the site of inflammation and subsequently appropriate activation and regulation. Cytokines play a critical role in this setting, since they regulate the proliferation and differentiation of T cells and determine the course of an inflammatory process by releasing pro- and anti-inflammatory cytokines. The chronic intestinal inflammation model has been presumably driven by a Th1-predominant immune response which results in accumulation and activation of T lymphocytes and macrophages. By orchestrating the inflammatory process, pro-inflammatory cytokines such as interleukin IL-12 and IFNγ are thought to promote the development of these diseases (25).

IL-12 plays a pivotal role in Thl T cell differentiation and induces naive T cells to produce IFNγ. Mannon et al. (26) prompted a successful clinical trial where intestinal inflammation was treated with a monoclonal antibody against IL-12, considering that previous studies demonstrated that the expression of IL-12, especially the IL- 12p40 subunit is greatly enhanced in patients with Crohn's disease. In our model, yoghurt feeding decreased the number of positive cells for both IFNγ and IL-12 in the large intestine of mice treated with TNBS after 13 days. This effect was observed with the previous or continuous yoghurt administration. With regard to IFNγ, increases in the number of cells that produce this cytokine were observed in mice given yoghurt in the basal sample and three days post-TNBS. This observation agrees with previous results where yoghurt feeding increased the number of IFNγ+ cells, but this fact was not related with inflammation in the intestine. It has been suggested that the large number of these positive cells in mice fed yoghurt could be related with the increase in immune cell numbers observed in the intestine. IFNγ would also be regulated by other cytokines such as IL-10 (10).

The recently described IL-17 expressing T helper cells (Th-17) may play a central role in T cell-mediated diseases including IBD (27). IL-17 has been found to be elevated in a variety of inflammatory conditions including IBD (28). In this sense, it is now a general concept that IL-17-stimulated production of inflammatory mediators is a key element in the inflammatory cascade in a variety of pathological conditions.

The study of the IL-17+ cells confirmed the anti-inflammatory effect of yoghurt in our model. Yoghurt feeding by itself did not increase this cytokine. In contrast to mice treated with TNBS which maintained the IL-17+ cells increased during all the experiment, the mice that received yoghurt decreased the cells positive for this cytokine in the samples taken after 7 days.

In addition, considering the results obtained with the pro-inflammatory cytokines where yoghurt administration induced a down regulation of the inflammatory response, IL-10 was studied as a regulatory cytokine. Berg et al. (7) showed the role of IL-10 in intestinal inflammation and carcinogenesis. Mice with a disruption of the IL-10 gene showed inflammatory changes in ceacum, colon and rectum with a high incidence of colorectal adenocarcinomas. IL-10 also participates in the normal tolerance to indigenous microbiota and its deficiency is related to inflammation (29).

In this study, the number of IL-10+ cells increased significantly in the mice fed with yoghurt compared to the TNBS group: This increase was more pronounced in continuous feeding than when yoghurt administration was suspended.

This last observation showed that IL-10 could be one of the mechanisms by which yoghurt can exert its anti-inflammatory effect. Interestingly, it was reported that the induction of colitis can be prevented by co-transfer of syngeneic CD4+CD45RBlow T cells (30). These CD4+CD45RBlow T cells are shown to exert their inflammatory effect via production of IL-10 and transforming growth factor-β (TGF-β).

The results obtained suggest that yoghurt could modulate the inflammatory cytokines IL-12 and IL-17 inducing down regulation of the immune cells involved in the inflammatory process and favouring IL-10 anti-inflammatory cytokine production.

In addition to this immunomodulatory capacity, another mechanism by which yoghurt could exert

the anti-inflammatory activity observed in our model would be through beneficial changes in the intestinal microbiota with increases in the bifidobacteria and lactobacilli populations.

The results obtained also showed the importance of continuous administration of yoghurt, even after the inflammation. This observation was related with the beneficial changes in the intestinal microbiota and the regulation of the immune system maintained with the continuous yoghurt consumption.

Studies of other mechanisms such as cells apoptosis and the innate immune cells involved in the anti-inflammatory effect of yoghurt are currently under investigation.

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