ANTITUMOUR ACTIVITY OF YOGHURT

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ABSTRACT

Several studies have demonstrated that fermented milk consumption decrease the incidence of colorectal cancer. Using a chemically induced murine colon cancer model it was reported that conventional yoghurt inhibits tumour development. In this model, the inflammatory immune response caused by the carcinogen (DMH) showed a great increase in IgG+ B cells, CD8+ T lymphocytes and in proinflammatory cytokines (TNFα and IFNγ). Yoghurt feeding inhibited tumour development by decreasing the inflammatory immune response and increasing the number of IgA+ cells, CD4+ T lymphocytes, cytokines such as Il-10 and decreasing NO radicals. Yoghurt also induced the apoptosis mechanisms. The local immune stimulation produced by yoghurt feeding increased monocytes/macrophages population and the cytokines release in the nodular tissue and in the Peyer's patches suggesting that these cells could be responsible for IFNγ and TNFα production. The enhancement of IL-10 found would favour the regulation of the immune response, not only in the inhibition model of the tumour growth, but also when yoghurt is given long term. The immune mechanisms involved by yoghurt to decrease the inflammatory immune response caused by the carcinogen were different to those observed with an antiinflammatory drug (indomethacin). Indomethacin did not increase immune infiltrative cell activity in the large intestine and the cytokine levels were diminished. Nitric oxide synthase enzyme determinations showed that in mice fed with yoghurt, the IFNγ enhancement was not related to inflammation, but to an immunomodulation. We demonstrated that the only single yoghurt supplementation was unable to inhibit tumour development in the initiation stage, however it inhibited the tumour growth (promption and progression) when it was administered cyclically after tumour induction. Cellular apoptosis increase observed could explain the importance for the TNFα levels found in the mice fed long term with yoghurt. The normal microflora has an important function in the intestinal inflammatory process preceding tumour development, lactic acid bacteria present in yoghurt play a role in this process since it has been shown that these bacteria and fermented milk products act on the microbial enzyme activities associated with colon carcinogenesis. This chapter will show that yoghurt can inhibit the promotion and progression of chemically induced colon cancer in mice through its antiinflammatory effect, cell apoptosis and by its immunomodulating properties.

ANTICARCINOGENIC ACTIVITY OF PROBIOTICS

Probiotics have been given credit for numerous health-promoting effects; they stimulate the immune system, possess anticarcinogenic and hypocholesterolemic properties, exert an antagonistic action against enteric pathogens and various intestinal disorders (Mital and Garg, 1995; Kato, 2000; Perdigón *et al.,* 2001) and play a role in the control of gastrointetsinal health. Their connection with animal and human cancers has been extensively reviewed (Rolfe, 2000; Hughes and Rowland, 2003). Probiotics such as lactobacilli and bifidobacteria in fermented or culture-containing dairy foods such as yoghurt may play a role in reducing the risk of colon cancer (Fernandes and Shahani, 1990; Braddy *et al.,* 2000; Wolloski *et al*., 2001). The increase of immune cells activity in the prevention of cancer by lactic acid bacteria (LAB) consumption has also been described (Kato *et al*., 1984; Hayashi and Ohwaki, 1989).

The intake of products containing viable LAB may lower the risk of colon cancer either directly by reducing procarcinogenic substances or indirectly by reducing the level of the enzymes (β-glucuronidase, azoreductase, nitroreductase, among others) that convert procarcinogens to carcinogens in the intestine (Goldin *et al*., 1980; Goldin and Gorbach, 1984; Fernandes and Shahani, 1990). The consumption of *L. acidophilus* in experimental animal models reduced the activity of fecal enzymes such as β-glucuronidase, azoreductase and nitroreductase (Goldin and Gorbach, 1976; 1980), this activity having been well correlated with the number of LAB in the intestine. The products of these enzymes are known to be mutagenic and carcinogenic. Goldin and Gorbach (1976) showed the relationship between diet and rat fecal bacterial enzymes implicated in colon cancer. Furthermore, the effect of the diet on various cancer types has been the subject of many reviews (Cummings and Bingham, 1998; Reddy, 1998).

A specific strain of *lactobacillus*, *Lactobacillus* GG, has been shown to reduce the incidence of tumours in carcinogen-treated laboratory rats, particularly during the early promotional stages of carcinogenesis and when animals are fed with a high fat diet (Goldin *et al*., 1996). Likewise, *Bifidobacterium longum* inhibited cell proliferation and the development of colon tumours in laboratory rats treated with a chemical carcinogen and fed with high fat diets (Singh *et al*., 1997, Abdelali *et al*., 1995; Reddy and Riverson, 1993). Sreekumar and Husono (2000) showed that *L. acidophilus* reduced fecal β-glucuronidase activity and the number of bacteria in the intestinal tract of rats.

Studies in humans have also shown that *L. acidophilus* supplements decrease the activity of fecal bacterial enzymes (β-glucuronidase and nitroreductase) that may convert procarcinogens to carcinogens (Goldin *et al*., 1980; Goldin and Gorbach, 1984). When healthy female adults in Finland supplemented their diets with yoghurt containing viable *Lactobacillus* GG for four weeks, fecal bacterial enzyme activity decreased (Ling *et al.,* 1994).

Another mechanism involved in tumour supression by LAB is the modulation of the host immune response (Perdigón *et al*., 2001).

FERMENTED MILKS AND PROBIOTICS. THEIR RELATIONSHIP WITH THE HOST IMMUNE SYSTEM

Live microbial food supplements added to improve the intestinal microbial balance, which benefically affect the host animal, are known as probiotics (Fuller, 1989). Schrezenenmeir and deVrese (2001) proposed the following definition, which confines the probiotic concept to effects produced by viable microorganisms; this concept is independent of the probiotic site of action and the route of administration: “A preparation of or a product containing viable, defined

microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and that exert beneficial health effects on this host." It is important to note that these definitions comprise not only preparations specifically designed to act as probiotics but also traditional yoghurt and other fermented products with LAB which have beneficial effects on the consumer.

Lactic acid bacteria, the most commonly used microorganisms in probiotic products, exert effects on the immune system of the host (Perdigon *et al.* 2001). Feeding with LAB and yoghurt stimulates the systemic immune response (macrophage function and number of immunoglobulin secreting cells) as well as the local immune response (IgA secretion into the intestine) (Perdigón *et al*., 1999; Vintiñi *et al*., 2000). Consequently, the consumption of fermented dairy products containing viable microorganisms such as yoghurt has increased. Besides enhancing the immune response, the consumption of fermented milks and LAB has been shown to increase resistance to neoplasia and infections (Kato, 2000). Gut associated lymphoid tissue (GALT) is under constant exposure to environmental antigens, the digestive flora being its main antigenic stimulus (Cebra, 1999). Another mechanism by which LAB is thought to promote health is through the normalization of intestinal flora which has been disturbed by disease or drugs (Kato, 2000).

Immunomodulation by LAB depends on the contact of these microorganisms with the lymphoid tissue which transitorily colonize the intestinal lumen. By this mechanism most LAB have the ability to survive in the gastrointestinal tract passage that can influence their immunogenicity (Schiffrin *et al*., 1997, Saxelin, 1996). LAB which survive the conditions of the gastrointestinal tract can adhere to the intestinal epithelial cells or M cells from Peyer's patches, stimulating the GALT and increasing the production of cytokines and antibodies (principally secretory IgA). (Perdigón *et al*., 2002a; Perdigón *et al*., 2001; Meydani and Ha, 2000).

Yoghurt has been defined in the Codex Alimentarius as a coagulated milk product that results from the lactic acid fermentation of milk by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus.* In this definition, in addition to the LAB present in the yoghurt, the peptides released during the fermentation process are important from the nutritional point of view. Many investigators have studied the therapeutic effects of yoghurt and LAB commonly used in yoghurt production against diseases such as cancer, infections and gastroinstestinal disorders. The immunomodulating and immunostimulating properties of yoghurt and fermented milks have also been well documented (Matar *et al.*, 2003).

The immunomodulation effects of yoghurt are in part caused by the bacteria contained in it. When viable or biologically active LAB enter the intestine, they can activate the GALT specific or nonspecific inmune responses as well as the systemic immune response (Meydani and Ha, 2000).

Perdigón *et al*. (1999) studied different LAB as well as the mechanisms involved in the mucosal immune system activation. Some LAB induced specific secretory immunity while others increased the intestinal inflammatory response. Vintiñi *et al*. (2000) demonstrated the importance of the LAB strain to be used. They studied, using BALB/c mice, the effect of different LAB (*Lactobacillus casei, L. acidophilus, L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus, L. plantarum, Lactococcus lactis* and *Streptococcus thermophilus*) on the intestinal mucosal response. They showed that not all LAB can be utilized as oral adjuvants and that their beneficial effects on the intestinal immune system are not limited for one bacterial genus or species. Consequently, the immunomodulating properties of LAB are strain specific.

Simulated commercial yoghurt feeding of BABL/c mice induced a marked immune cell infiltration with plasm cells and lymphocytes prevalence (Perdigón *et al*., 1994). Yoghurt feeding for 7 days increased the number of IgA+ cells in the small and large intestine of mice, but IgM+

and IgG+ cells did not increase (Perdigón *et al.*, 1998). Macrophage numbers were higher in regard to non-treatment control group, but they did not show increased activity. The above observations explain why yogurt does not over stimulate the undesirable inflammatory response (Valdéz *et al*., 1997).

In addition to LAB, yoghurt possesses other non bacterial components produced during fermentation which can contribute to immunogenicity and to other properties like its antitumour activity of yoghurt, which is related to its immunological modulator potential.

Peptides and free fat acids released during fermentation were shown to increase the immune response. In this way, peptidic fractions liberated during milk fermentation with a strain of *Lactobacillus helveticus* stimulated the immune system and inhibited the growth of an immuno dependent fibrosarcoma in a mice model (LeBlanc *et al*., 2002). The peptidic profiles of milk proteins were significantly different after fermentation by LAB, suggesting that microbial proteolysis could be a potential source of bioactive peptides (Matar *et al*., 1996). Milk fermented with *Lactobacillus helveticus* R389, a bacterium with high protease and peptidase activity, exerted an antimutagenic effect while a mutant strain, deficient in proteolytic activity, did not (Matar *et al*., 1997). In a similary way, milk fermented with the proteolytic strain increased the number of IgA+ cells in the small intestine as well as in the bronchi of mice, but fermented milk obtained with the mutant strain did not show the same *in vivo* results (Matar *et al*., 2001).

Fractions separated by dialysis from yoghurt showed tumour inhibition in *in vivo* murine assays (Ayebo *et al*., 1982). Certain soluble components produced by LAB during milk fermentation could be used to prevent certain malignant gastrointestinal pathologies (Biffi *et al*., 1997). The filtrate of yoghurt was reported to increase IFNγ production by natural killer cells (de Simone *et al*., 1986).

YOGHURT AND COLON CANCER. A STUDY OF THE IMMUNE POPULATIONS AND CYTOKINE PROFILES INVOLVED.

Colon cancer inhibition by yoghurt was studied in an experimental model using BALB/c mice (Perdigón et al. 1998). Animals were fed with yoghurt for 10 consecutive days (the dose with the best effects of yoghurt on the intestinal immune system). The colon tumour was chemically induced with dimethylhydrazine (DMH) and the animals were given yoghurt cyclically again after tumour induction (for ten consecutive days followed by a one week break and then again for ten days) until the end of the experiment (six months).

Yoghurt feeding inhibed tumour growth (yoghurt-DMH-yoghurt group) (Figure 1A). In this experimental model, a large inflammatory immune response was observed during the tumour development in the large intestine in the mice treated only with DMH (tumour control or DMH group). The inflammatory response was observed histologically by identification of the immune cells. The increase in IgG-producing B cells and CD8+ T lymphocytes (Table 1) would point to their participationin the inflammatory process. The increased number of CD8+ cells enhanced the cytotoxic immune response with an increase in the inflammatory immune response. Furthermore, the IgG+ cells determined could increase the inflammation by the cytolytic activity of IgG through the complement system. When the DMH injected mice were fed with yoghurt, an increase in the number of IgA-secreting cells and CD4+ T lymphocytes (Table 1) in the lamina propria of the large intestine with a decrease in the IgG+ and CD8+ cells was observed (Perdigón *et al*., 1998). The antitumour activity observed with yoghurt feeding could be caused either by the immunomodulatory capacity of the LAB (Perdigón and Oliver, 2000) or of yoghurt (Perdigón *et al*., 1994). The increase in the number of IgA+ cells but not of IgG+ cells in the large intestine of the mice fed with yoghurt could limit the inflammatory response, since IgA is considered as an important barrier in colonic neoplasia (Isaacson, 1982).

Since mice were fed with yoghurt for long periods, another experimental group (yoghurt group) was added in order to determine the effect of long term feeding with the fermented product. The histological studies showed that a large number of immune cells infiltrated the lamina propria of the large intestine in this group (figure 1C). However, CD4+ and CD8+ T lymphocytes did not increase in comparison with non-treatment control mice (de Moreno de LeBlanc *et al.*, 2004) while long term yoghurt feeding increased IgA-secreting cells, being remarkable in the fourth and sixth months (Table 1). All these results suggest that the antitumour activity of yoghurt may be exerted by its ability to mediate the down regulation of the inflammatory response induced by the carcinogen, since it is known that inflammation is a risk factor for several types of cancer (Prescott and Fitzpatrick, 2000).

The studies concerning the cell population in this model allowed to suggest the possible antinflammatory role of yoghurt to account for its antitumour activity (de Moreno de LeBlanc et al., 2004). Since cytokines are the biological messengers for the regulation and modulation of the immune response, they are a pontential target for the study of the mechanisms induced by LAB or yoghurt to modulate the immune response.

Different cytokines were studied using immunohistochemic or immunfluorescent methods on large intestine slices from the different groups of mice treated with DMH, yoghurt-DMH-yoghurt or yoghurt. TNFα (tumour necrosis factor) was studied due to the monocyte/macrophage prevalence in our experimental model and because these actived cells release it. TNFα has proinflammatory properties but can also mediate cellular apoptosis (Sellers and Fisher, 1999).

IFNγ was selected at first as the Th-1 lymphocyte population marker. The number of CD4+ cells reported for the mice fed with yoghurt after DMH treatment could account for the decrease in the inflammatory response through cytokines release for CD4+ population (IL-4, IL-10, IFNγ).

Although IFNγ is a proinflammatory cytokine, it is able to kill colonic epithelial cells (Numata *et al*., 1991).

It was observed that the proinflammatory cytokines (TNFα and IFNγ) were increased in the cells from the large intestine of tumour control mice (DMH group) and in the yoghurt-DMH-yoghurt group (Table 1). Yoghurt feeding itself also produced high levels of these proinflammatory cytokines (Perdigón *et al*., 2002b).

In mice injected with DMH, the nodular infiltrates in the large intestine showed an enhacement in their number and size. The cells from these nodules were important to study the effect on the immune responses of the DMH and the yoghurt-DMH-yoghurt groups (de Moreno de LeBlanc and Perdigón, 2003). In all the assayed periods, up to six months, yoghurt feeding increased TNFα producing cells in comparison with the tumour control group. These cells comprise non-adherent (fibroblast, mast cells and some T and NK cells) as well as adherent (macrophages / monocytes) cells (Feghali and Wright, 1997). In our experimental model, adherent cells produced large amounts of TNFα than non-adherent. The number of IFNγ+ cells also increased with yoghurt supplementation. This cytokine is produced mainly by cells belonging to non-adherent populations such as T cells and NK cells (Feghali and Wright, 1997). IFNγ production by other cells such as macrophages and dendritic cells, which are considered adherent cells, was reprted recently (Frucht *et al.*, 2001). In our study, in addition to non-adherent cells, we detected IFNγ (nearly 50%) in the adherent cells. All these observations show that yoghurt stimulates the activity of infiltrative cells (adherent and non adherent), which increased cytokine production, necessary for tumour resolution. As previously suggested by de Moreno de LeBlanc *et al.* (2004), adherent cells play an important role in the immune responses observed in our tumour model. They would also be accompanied by activated non-adherent cells.

The above results led us to study the effect of long term yoghurt feeding on the cytokine released from Peyer's patches cells in order to evaluate its effect on the intestinal immune system, since Peyer's patches are inductor sites of the mucosal immune reponse. TNFα and IFNγ significantly increased in mice fed with yoghurt for ten days, after which time values remained constant. TNFα increase was due to the adherent population, whereas both adherent and non-adherent populations were responsible for IFNγ production (de Moreno de LeBlanc and Perdigón, submitted). Yoghurt only stimulated cytokine production by Peyer's patch cells at the beginning of yoghurt supplementation, after which a basal cytokine level was maintained in these cells.

We analyzed other regulatory cytokines (IL-10 and IL-4) that could be involved in the immune response of the mice fed with yoghurt since, unlike the DMH group, the increased proinflammatory cytokine positive cells were not related to the inflammation and tumour development observed in those animals.

IL-10 and IL-4 are associated with activated Th-2 lymphocytes but IL-10 can also be produced by other cell populations such as macrophages and dendritic cells.

In different experimental models, TNFα and IL-10 were demonstrated to have opposite effects (Bogdan *et al*., 1992). The balance betweeen TNFα and IL-10 could modulate the effector function of macrophages and cellular apoptosis. Furthermore, IL-10 is important as a regulator in the intestine. Berg *et al*. (1996) demonstrated the IL-10 role in intestinal inflammation and carcinogenesis. Mice with a disruption of the IL-10 gene showed inflammatory changes in caecum, colon and rectum with a high incidence of colorectal adenocarcinomas. IL-10 also participates in the normal tolerance to indigenous bacterial flora and its lack is related to inflammation (Sydora *et al*,. 2003).

In our model, the number of IL-10+ cells increased significativelly in all the samples from the three experimetal groups (DMH, yoghurt-DMH-yoghurt and yoghrut) (Perdigón *et al*, 2002a). It is important to remark that yoghurt feeding always produced more IL-10+ cells in the yoghurt-DMH-yoghurt group than in the DMH group.

IL-4 plays a significant role both in controlling cell growth and in modulating the immune response (Chang *et al*., 2000). This cytokine has antagonist functions to IFNγ and appears to possess certain antiinflammatory properties, IL-4 can inhibit the production of several proinflammatory cytokines such as IL-1, IL-6, IL-8 and TNFα (Feghali and Wright, 1997). Patton *et al.* (2002) showed that *Schistosoma mansoni*-infected IL-4 deficient mice (IL-4 $^{-/-}$) developed a Th-1 response with excessive and prolonged production of nitric oxide (NO) associated with enhanced IFNγ production and IL-4 is also associated with immune modulation in some tumour models (Nishihori *et al*., 2000).

The increase in IFNγ values led us to measure IL-4 production since we thought that IL-4 would modulate the production of IFNγ. We determined that, among the groups studied, IL-4 was the cytokine that presented the most remarkable differences. The number of IL-4+ cells increased at the beginning of the experiment in the yoghurt-DMH-yoghurt group; IL-4 probably exerts control over the inflammation, which helps in the non-development of the tumour; afterwards, the number of IL-4+ cells was similar to those obtained with the non-treatment control group (figure 2). IL-4+ cells only increased at the end of the experience (six months) in the DMH group, in which time the mice presented important lesions in the large intestine and development of the tumour. In this group it is possible that the great alterations produced in the intestine together with the high levels of cytokines such as IFNγ and TNFα increased the IL-10 levels to regulate the immune response. This could not by itself control the inflammatory response induced by the

carcinogen so that the IL-4 regulatory cytokine was released. However, at this stage of tumour evolution (fourth and fifth months), tumour growth could be not reverted.

Yoghurt feeding by itself did not increase IL-4+ cells, although it increased IL-10+ cells in the large intestine (figure 2). These results suggest that yoghurt could modulate the immune response; it stimulates cytokine production when this is required, or induces down regulation of the immune cells to avoid exacerbation of the immune response. This effect would occur mainly through IL-10, which showed increases in the tissue during all the periods assayed.

The increase in cell number and cytokines production in mice after long term feeding with yoghurt led us to study the Bcl-2 protein as a marker of cell activity and mitosis. This protein is a measure of cell survival due to its antiapoptotic activity (Sellers and Fisher, 1999). We found that the number of Bcl-2(+) cells increased in the large intestine of mice after long term yoghurt feeding as well as in the samples obtained after assay for two and three months in the yoghurt-DMH-yoghurt group. The number of Bcl-2(+) cells did not increase in the DMH group except in the sixth month, when the animals had developed tumour (de Moreno de LeBlanc *et al*., 2003). The increase in Bcl2(+) cells observed in the group of mice after long term yoghurt feedding may be related to cell infiltration and survival caused by the fermented milk and would explain the enhancement in the number of cytokine positive cells observed in this group of animals.

We were able to study another role of CD4+ T lymphocytes due to their increase in the DMH group when yoghurt was added to the diet. There is now evidence that CD4+ T cells which express the CD25 marker play a critical role in immune regulation through IL-10 production (Read and Powrie, 2001). There are discrepancies regarding the regulatory activity and the CD25 marker. Curotto de Lafaille and Lafaille (2002) reported that CD4+CD25- T cells are as effective as CD4+CD25+ T cells in controlling T-cell mediated diseases. We analyzed the CD25 marker of the large intestine in the treatment periods in which the number of IL-10+ cells was highest. We

found that the CD25+ marker had increased in some samples (de Moreno de LeBlanc and Perdigón, submitted). The results obtained showed that the CD4+CD25+ T regulatory cells were not the only cell population responsible for the increased levels of IL10 observed in our tumour model.

In order to determine the importance of CD4+ T cells enhancement in the yoghurt-DMH-yoghurt group, we decided to study the T cell receptor (TCR). Infiltrative lymphocytes in certain solid tumours are characterized by the γ/δ chains presents in the TCR. These lymphocytes would produce certain molecules in the tumour site which would contribute to the antitumour immune response (perforines and cytokin es). The IFNγ released by γ/δ (+) TCR lymphocytes present in the tumour is one of the cytokines responsible for this action (Ferrarini *et al*., 2002). In our experimental colon cancer model, as mentioned above, IFN+ cells increased in all the group assayed; thus the study of the TCR will permit to know whether these T lymphocytes play an important role in the antitumor immune response exerted by yoghurt through IFNγ.

T γ/δ (+) and α/β(+) cells were studied in the different experimental groups. We did not observe any significative increases in either α/β(+) or γ/δ (+) TCR in any of the groups, so that γ/δ (+) TCR cells would not be the mainl source of the IFNγ enhancement observed.

It has been reported (Perdigon et al., 2000) that *Lactobacillus delbruekii* subsp *bulgariccus* and S*treptococcus thermophilus* interact with the small intestine cells of mice but not with those of the large intestine. Yoghurt preparation is a symbiotic interaction between both bacterial species where the starter culture is a strain pools of each bacterial species. Consequently, we analized in mice the interaction between yoghurt starter bacteria or bacterial antigens and the intestine using a fluorescent bacterial suspension grown under conditions similar to commercial yoghurt. We

determined that fluorescent cells from the starter culture were able to interact with Peyer's patches and with the large intestine (figure 3). This finding allowed us to understand why the mice fed with a long term yoghrut suplemented diet showed an increased cell activity (Bcl-2(+) cells) and cytokine positve cells. Yoghurt bacteria interact with Peyers's patches cells (inductor sites of the gut immune response) and the effect on the large intestine could be mediated by direct interaction with the immune cells or indirectly by cytokines produced in that inductor site of the intestine immune system which exert their functions at a distance (Barret, 1996).

This study allowed us to demonstrate that yoghurt exerts a certain interaction directly with the large intestine cells, reinforcing or increasing the responce produced in the immune inductor site (Peyer's patch). This would be further evidence that yoghurt can have an effect on pathologies affecting the mucose of the large intestine.

RELATIONSHIP BETWEEN INFLAMMATION AND TUMOUR

The association of chronic inflammation with several malignant diseases has been reported for a long time (Prescott and Fitzpatrick, 2000). There is also evidence that this relationship would be mediated by cytokines (Feghali and Wrigth, 1997) or by reactive oxygen species generated by inflammatory phagocytes that can cause injury to target cells, contributing to cancer development (Weitzman and Gordon, 1990). Examples of the above include intestinal cancer after intestinal chronic inflammation (Korelitz, 1983; Collins *et al*., 1987) and gastric cancer after atrophic gastritic (Correa, 1988). In addition to this, it seems clear that some carcinogens (such as tobacco in lung tumours) are also associated with a chronic inflammatory process. (Weitzman y Gordon, 1990).

As regards the relationdhip between inflammation and tumour the oncology has recently, recognized that several drugs such as the inhibitors of the cyclooxigenase enzyme (COX) as the antiinflammatory drugs (NSAIDs), can delay or prevent certain cancers.

The target for the study of NSAIDs is now COX-2, an inducible isoenzyme of cyclooxygenase which would be induce in the inflammatory sites by different factors such as cytokines.

A number of reports have indicated that the metabolites of arachidonic acid, especially prostaglandins of the E series, are involved in the initiation, promotion and progression of tumoral processes (Fischer, 1985; Trosko *et al*., 1985; Fletcher, 1989). Several studies have demonstrated the beneficial effect of cyclooxygenase enzyme inhibitors on the therapy of a variety of tumours and metastases (Fulton, 1984; Lala *et al.*, 1986; Reddy *et al.,* 1987). In a murine fibrosarcoma, the antitumoral activity of antinflammatory drugs (piroxicam, indomethacin and aspirin) was correlated with the recovery of peritoneal macrophages activity and peripheral blood leukocytes level (Valdéz and Perdigón, 1991).

On the basis of the thow previous results obtained in which the antitumour effect of yoghurt on an induced colon cancer could be explained by a regulation of the inflammatory immune response exerted by the DMH carcinogen, with a significant increase in the activity and survival of the cells by long term feeding with yoghurt, we analized the effect of an NSAID such as indomethacin on the inhibition of colon cancer to compare it with the antitumour activity of yoghurt. A new group of mice to be treated with indomethacin was added (DMH-indomethacin group).

Histological observations showed that, during indomethacin administration, the immune cells infiltrated into the large intestine were smaller (de Moreno de LeBlanc *et al*., 2004), different from those observed with the yoghurt feeding, where a great increase in the number of immune cells infriltrating the lamina propria (yoghurt-DMH-yoghurt group) was found. However, no

changes in the tissue was detected in relation to the carcinogen group. The cellular infiltration also occurred in the large intestine of long term yoghurt fed mice, suggesting that these infiltrative cells would play an importat role in the antitumoral and antiinflammatory activity of yoghurt.

During the fourth month of the indomethacin treatment, the histology of the large intestine from this group of mice was similar to the non-treatment control group. When indomethacin administration was stopped due to cachexia in the animals, the tumour developed with the same characteristics as in the DMH group in the sixth month (de Moreno de LeBlanc *et al*. 2004).

This last observation showed different mechanisms for indomethacin and yoghurt, since when the yoghurt feeding was stopped at the end of the experiment (six months), the animals from the yoghurt-DMH-yoghurt group, which were observed until the ninth month did not develop tumour.

CD4+ and CD8+ T lymphocytes were study in the large intestine from DMH-indomethacin group and another difference with the yoghurt feeding (yoghurt-DMH-yoghurt group) was observed. Mice treated with indomethacin increased the number of IgA+ cells only during the lst three monts of the study, but they did not show a different balance between CD4+ or CD8+ populations as was reported for yoghurt-DMH-yoghurt and DMH groups when the inflammation decreased or increased (Table 1).

Another difference between indomethacin and yoghurt was found during the proinflamatory cytokines evaluation. Mice injected with the antiinflammatory drug presented fewer infiltrative cells in the large intestine with a low number of positive cells for TNFα and IFNγ. When the drug treatment was stopped, the cellularity and the proinflammatory cytokines increased and the tumour grew (Table 1).

For the purpose of demonstrating that the proinflammatory cytokines increase in the large intestine from mice fed to yoghurt was not related with the development of a gut inflammatory response and that this cytokine response was being regulated, the nitric oxide synthase enzyme was studied.

The inducible oxide nitric synthase enzyme (iNOS) was evaluated in slices from the large intestine of different groups of mice (Table 1). INOS, which produces nitric oxide from L-arginin, is induced during the course of the immune response by microbial products and/or cytokines and plays a role in the antimicrobial effector mechanism of macrophages (Bogdan *et al*., 2000). One of the Th1 effector mechanisms mediated by IFNγ is iNOS induction, where IL-4 plays an important role as a regulator (Patton *et al*., 2002).

It was demonstrated that tumour bearing mice (DMH group) presented high amounts of iNOS+ cells, suggesting an increase in nitric oxide production (NO) by these cells. The iNOS enzyme synthesis would be induced by IFNγ which was increased in the intestinal tissue from this group. In the group of mice injected with DMH and fed with yoghurt, when the inflammation decreased, the iNOS+ cells also decreased. Long term yoghurt adminstration showed an iNOS+ cells number similar to that of the non-treatment control group throughout the experiment. The DMH-indomethacin group showed increased number of iNOS+ cells at the beginning of the antiinflamamtory treatment and at the end of the study, when the tumour grew. iNOS+ cells increase agreed with the IFNγ+ cell increase observed in the DMH-indomethacin group.

The lack of iNOS enzyme induction in the yoghurt-DMH-yoghurt and yoghurt control groups demonstrated the way in which yoghurt would regulate the immune system by modulating the inflammatory response. In spite of the increased number of IFNγ+ cells, these animals did not increase NO production and so did not present tumour, but only cellular infiltration. We thought

that the large number of positive cells for in mice fed with yoghurt would be related to the increase in the number of immune cells observed in the intestine; IFNγ would be regulated by other cytokines such as IL-10.

These results allow us to suggest that the immune mechanisms by which yoghurt operates would be different for those induced with the antiinflammatory drug indomethacin, which did not show an increased activity of the infiltrative immune cells in the large intestine, where cytokine levels were lower than in the others groups and iNOS diminution was only evident during treatment. Yoghurt exerted its antitumour activity by an antiinflammatory activity, down modulating the immune response principally through IL-10.

STAGES OF COLON CANCER IN WHICH YOGHURT ACTED.

Most of the antiinflammatory drugs studied by different authors and using different models, exert their antitumor activity during the early stages of tumour development. It is known that the development of colon cancer presents a sequence of events that occurs in definable steps (initiation, promotion and progression). In the results described above mice fed with yoghurt before and after DMH injections (yoghurt-DMH-yoghurt group) did not develop colon cancer throughout the period assayed (six months) in contrast with the tumour control group, which showed an intestinal tumour in the fifth or sixth month. In order to find out during which stage of the tumour process yoghurt acted (initiation, promotion or progression of tumour growth), we studied whether previous feeding with yoghurt was sufficient by itself to reach the regulatory immune response observed, or whether cyclical administration of yoghurt was necessary to prevent the effect of the DMH carcinogen.

Histological studies from the large intestine showed that previous yoghurt feeding for 10 days before DMH injections only delayed tumour appearance (de Moreno de LeBlanc and Perdigón, 2004). Tumour tissue with the same characteristics as the DMH group was observed in the seventh month. Cytokines studied in the large intestine of these mice showed that IFNγ and TNFα increased during all the periods assayed. IL-4 increased in the first months in the yoghurt-DMH-yoghurt group. The number of IL-4+ cells decreased when yoghurt feeding was stopped while the increase in IL-10 persisted in the large intestine cells after that time. In the same way as in the tumour control group, IL-4+ cells increased again at the end of the experience; however, even when the number of IL-10+ cells increased, it was not enough to stop the inflammation and the tumour developed.

To study the effect of yoghurt feeding in the promotion and progression of the tumour, a group of animals did not receive yoghurt previous to DMH injection but were fed cyclically with this fermented milk after tumour induction following the same protocol as the yoghurt-DMH-yoghurt group. These animals presented amelioration in the large intestine when the yoghurt was cyclically added to the diet after tumour induction with the carcinogen. This group of mice did not develop tumour and it was very similar to the yoghurt-DMH-yoghurt group previously described. In both groups, the number of immune cells increased in the lamina propria of the large intestine while cytokine production was similar to the one in the yoghurt-DMH-yoghurt group.

These results demonstrate that yoghurt exerts its antitumour activity by inhibition of tumour progression and that this effect is achieved by long term cyclical yoghurt consumption

OTHER MECHANISMS INVOLVED IN THE ANTITUMOUR EFFECT OF YOGHURT

The mechanisms of apoptosis or programmed cell death in the inhibition of tumour progression are well documented (Butler *et al*., 1999). Apoptosis is a complex and active cellular process where individual cells are triggered to undergo self-destruction in a manner that will neither injure neighboring cells nor elicit an inflammatory reaction. The colonic epithelium is a tissue with a high cell turnover rate. The balance between cell proliferation and cell death is important to maintain the length of the crypts. A disturbance in this balance may lead to tumour development (Hao *et al*., 1998), since the disruption of this type of regulation is a characteristic of the tumours.

Considering that cytokines such as TNFα could be involved in certain apoptotic pathways (Sellers and Fisher, 1999), and that an enhancement of this cytokinewas observed in our experimental model, apoptosis induction (Table 2) and the relationship between mitosis and apoptosis were studied in mice injected with DMH and in mice injected with DMH and fed with a diet supplemented with yoghurt.

An increase in mitosis during the first four weeks of tumour was observed in the animals treated with the carcinogen. They presented an increase in the number of apoptotic cells during the second and third months in relation to the non-treatment control. These values decreased when the tumour grew. In the mice from the yoghurt-DMH-yoghurt group a moderate cell proliferation with a significant increase in the number of apoptotic cells was reported (Rachid *et al*., 2002).

Yoghurt feeding previous to carcinogen injection increased apoptosis until the fourth month (de Moreno de LeBlanc and Perdigón, sumitted). This observation and the enhancement of regulatory cytokines induced by the previous yoghurt diet could explain the delay in the tumour appearance in this mice group. The behaviour of the mice that received yoghurt only after DMH injections was similar to the yoghurt-DMH-yoghurt group, where the number of apoptotic cells increased throughout the experience (Table 2).

Long term feeding with yoghurt increased the number of apoptotic cells, showing that yoghurt favours cellular apoptosis in the large intestine (table 2). This fact demonstrates the importance of high TNFα levels in the mice given only yoghurt as a dietary supplement, since yoghurt could exert a control in the intestine by modulating the immune response and stimulating cell apoptosis, thus preventing the possible harmful effects of prolonged yoghurt comsumption (Perdigon *et al*. 2003).

We cannot ignore the fact that the intestinal inflammatory process preceding tumour development may be due to changes in the epithelial cells induced by the carcinogen that enters the intestine as glucuronide. The normal microflora has the role of deconjugating this compound synthesized by gut microorganisms. Lactic acid bacteria present in yoghurt could play a role in this process. Many authors have shown the influence of ingested LAB and fermented milk products on the activity of microbial flora enzymes associated with colon carcinogenesis. Bifidobacteria and lactobacillus have these enzyme activities lower than bacteroides, clostridia and enterobacteriaceae.

Since it has been demonstrated that yoghurt bacteria interact in the large intestine and can affect the intestinal microflora, it is important to find out in our model whether other mechanisms such as a decrease in the activity of certain enzymes in the intestine can also be involved in the antitumour activity of the yoghurt.

It was demonstrated that long term yoghurt feeding maintained β-glucuronidase and nitroreductase activities similar or lower than in the non-treatment control group (figure 1 and 2), and were different than the values obtained with the carcinogen DMH, which were increased, contributing in this way to its mutagenic power.

Mice injected with DMH which were fed cyclically with yoghurt presented enzyme activity levels lower than the tumour control group. This last observation could explain previously reported histological differences between both groups (Perdigón *et al.* 1998). It is important to note that the decrease of these enzyme activities was observed in the samples before and after that the cell were broken with the glass beads, showing that yoghurt feeding decreases the levels of the enzymes in the intestinal fluids and prevents their induction in the interior of the cells.

It was important to compare the effect of yoghurt and the non-bacterial fraction of this fermented food on the large intestine enzymes, because yoghurt possesses not only lactic acid bacteria but also other substances released during milk fermentation. These products can inhibit enzyme activities showing another cancer preventing mechanism of yoghurt. In the present work, the variations of the β-glucuronidase and nitroreductase activities observed in mice fed whole yoghurt were not observed in the animals given yoghurt supernatant. Similar effects to those observed with milk were observed, where the enzyme activities were higher than the non-treatment control in some periods of the cyclical feeding. The last observation allowed the speculation that yoghurt bacteria would be involved in the diminution of the procarcinogenic enzyme levels reported in this paper.

Even when there are many evidences of antiinflammatory and immune modulator properties of the yoghurt, the results of this study implicate another mechanism by which yoghurt could exert the antitumour activity observed in our murine model: at the level of normal intestinal flora, by reducing of the gut enzyme activities which are involved in the colon carcinogenesis.

CONCLUSIONS

The different studies carried out with the model of colon cancer inhibition through cyclic yoghurt feeding demonstrate that yoghurt modulates the immune system response and exerts its antitumour activity through its antiinflammatory capacity. Yoghurt induces apoptosis and IL-10

production in the large intestine. This effect would be obtained by long term cyclic yoghurt consumption, which inhibited promotion and progression in our experimental intestinal tumour.

In addition to this immunmodulator capacity, another mechanism by which yoghurt could exert the antitumor activity observed in our model would be through yoghurt bacteria interaction with the intestinal cells. These bacteria could induce decrease in the certain enzyme activities involved in the development of tumours in the intestine.

Numerous mechanisms in which the immune system plays a role can be involved in the antitumour activity of yoghurt, which would be mediated by the bioactive peptides released during fermentation and by the microorganisms used as feremented starters. Our studies have demonstrated the immunoregulatory and antiinflammmatory effect of yoghurt on the mucosal immune response as well as its capacity to induce cell apoptosis and thus prevent tumour growth.

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TABLE 1. Comparative study of the cell populations, proinflammatory cytokines and iNOS enzyme

EXPERIMENTAL GROUP	Period of treatment	IgA	CD4	CD8	TNFα	IFNγ	iNOS
DMH	2 months	60*±8	20±3	50*±2	68*±4	86*±15	23*±5
	3 months	60*±8	22±3	50*±3	97*±15	162*±15	19±6
	4 months	70*±7	ND	ND	155*±22	140*±17	41*±5
	5 months	40±4	36±2	80*±4	123*±16	134*±3	22±4
	6 months	ND	ND	ND	125*±10	99*±22	25*±5
Y-DMH-Y	2 months	100*±10	40±2	30*±2	64*±8	105*±6	15*±5
	3 months	130*±8	82*±5	40*±2	78*±11	115*±10	15*±6
	4 months	100*±7	ND	ND	57*±18	121*±27	14*±4
	5 months	50±3	35*±2	10±1	130*±21	144*±9	12*±3
	6 months	ND	ND	ND	75*±10	86*±6	12*±2
Yoghurt	2 months	ND	ND	ND	ND	ND	ND
	3 months	84±17	29±8	12±4	50*±7	100*±10	19*±5
	4 months	108*±12	22±4	14±4	57*±8	112*±9	11*±2
	5 months	79±10	21±2	15±6	81*±9	129*±14	14*±3
	6 months	95*±13	17±4	10±3	101*±14	86*±17	12*±2
DMH-indomethacin	2 months	76±14	41*±6	24*±5	42*±9	69*±9	24*±5
	3 months	62±12	31*±5	20*±7	20±8	40*±8	16±5
	4 months	106*±24	22±4	17±6	27±10	23±5	18±3
	5 months	125*±3	18±3	16*±3	16±7	24±6	8±2
	6 months	114*±22	32*±7	22*±5	26±7	35*±8	20*±2
Yoghurt basal	10 days	84±11	19±3	10±3	29±3	25±3	8±2
Non-treatment control		65±5	20±3	11±2	17±2	21±2	10±2

Results are expressed as number of positive cell for the correspondient cellular marker, cytokine or enzyme counted in 10 fields of vision as seen at 1000X magnification using a fluorescence light microscope. * = significant differences of each group comparing with non-treatment control. $P < 0.001$. Y-DMH-Y= yogurt-DMH-yoghurt; ND = Not determined.

TABLE 2. Cellular apoptosis determination

Experimental group	Period of treatment	N° apoptotic cells/10 fields
DMH	1 month	ND
	2 months	43±5 *
	3 meses	26±2
	4 months	47±6 *
	5 months	ND
	6 months	ND
Y-DMH-Y	1 month	ND
	2 months	ND
	3 months	110±10 *
	4 months	60±9 *
	5 months	27±7
	6 months	33±6
Yoghurt-DMH	1month	52±12 *
	2months	60±14 *
	3months	82±16 *
	4months	55±11 *
	5months	13±2
	6months	6±2
DMH-yoghurt	1month	24±6
	2months	82±13 *
	3months	61±19 *
	4months	177±8 *
	5months	19±2
	6months	32±4
Yoghurt	1month	ND
	2months	ND
	3months	114±13 *
	4months	50±9
	5months	99±12 *
	6months	62±11 *
Basal yoghurt	10 days	34±6
Non-treatment control		25±3

The results are expressed as means ± SD of the number of apoptotic cells counted in ten fields of vision at 400X of magnification (Cells / 10 fileds) using slices from large intetsine.
* = significant differences between the sample of test groups and the non-treatment control group. P< 0.001.Y-DMH-Y= yogurt-DMH-yoghurt; ND = Not determined.

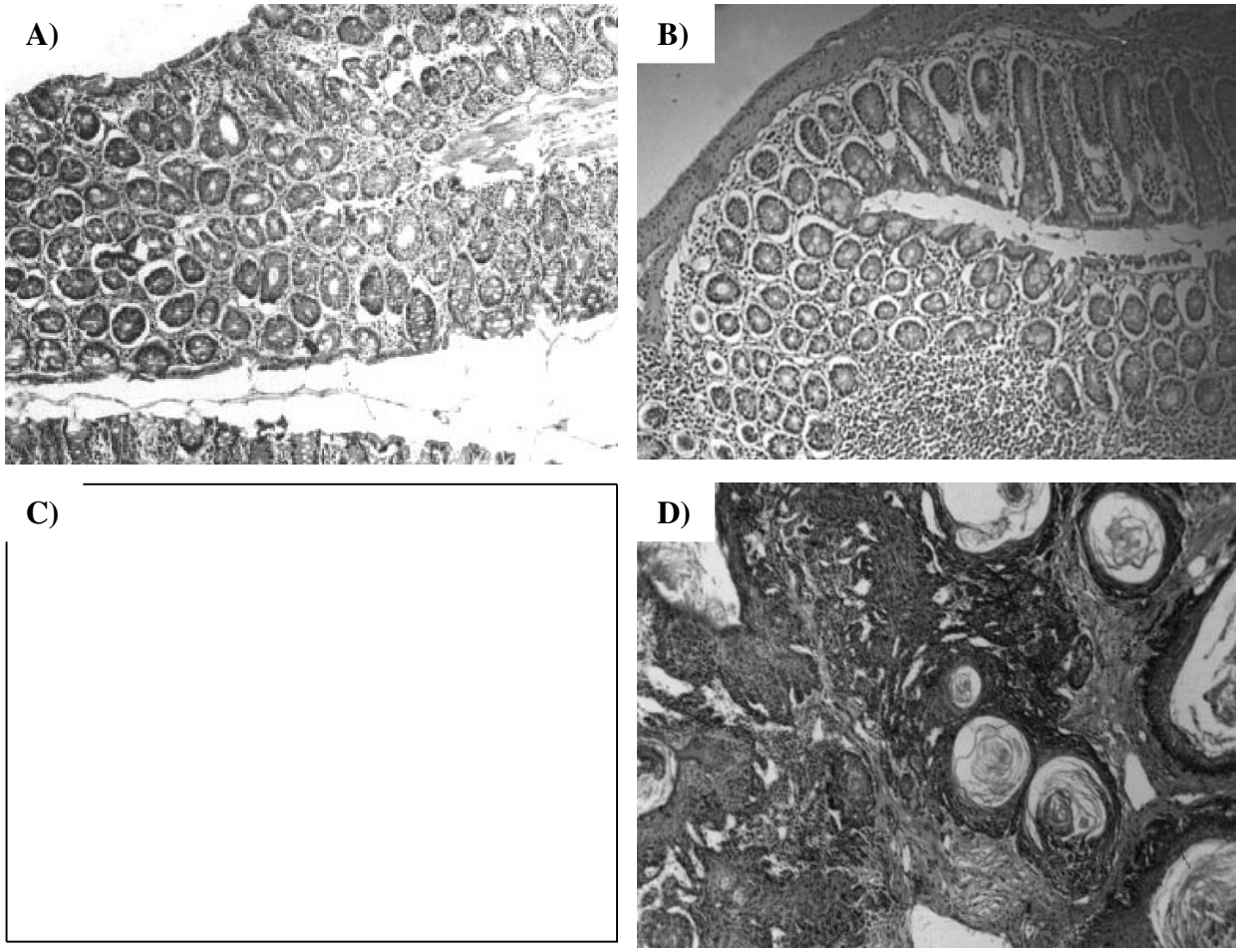

Figure 1

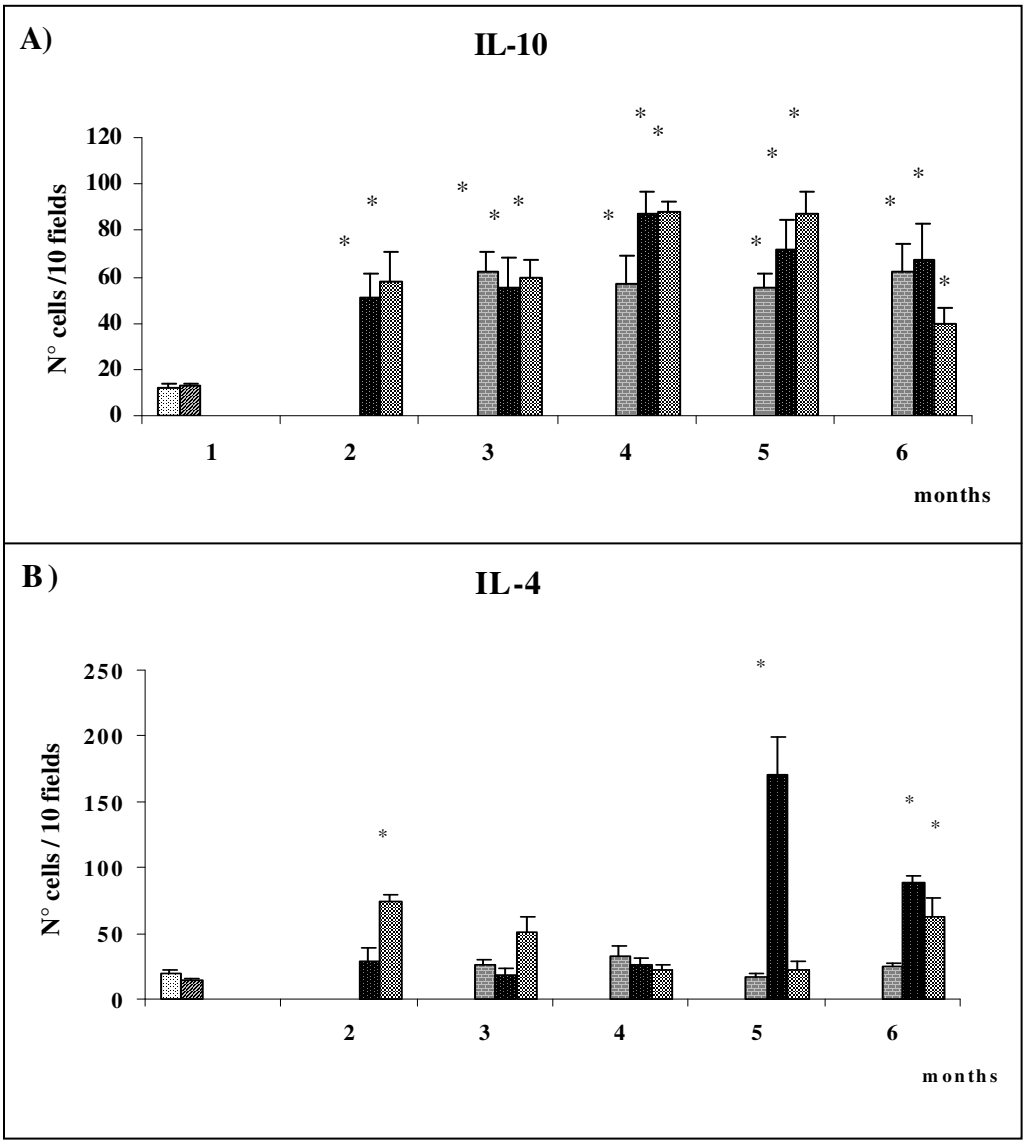
A)
IL-10
N° cells /10 fields
120
100
80
60
40
20
0
1
2
3
4
5
6
months
B)
IL-4
N° cells / 10 fields
250
200
150
100
50
0
2
3
4
5
6
months

Figure 2.

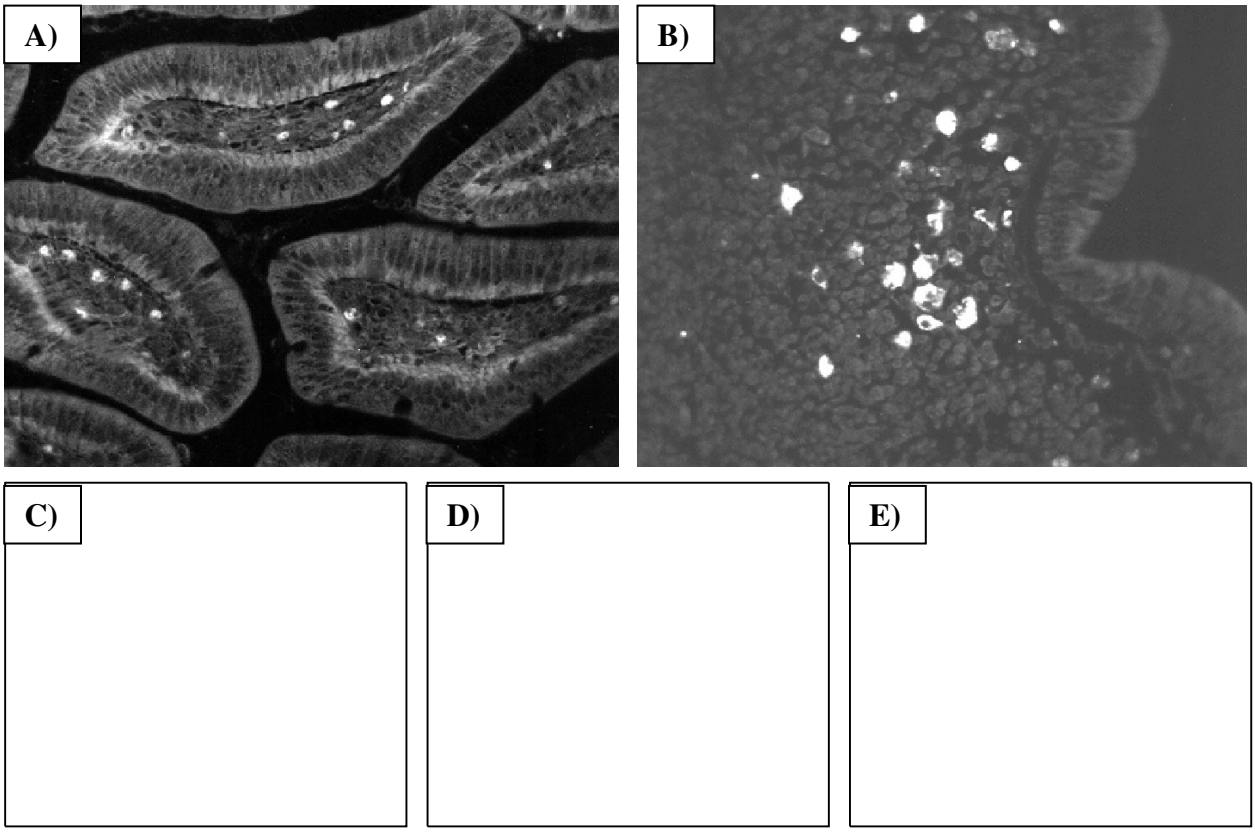

Figure 3.

FIGURE LEGENDS

Figure 1. **Histological study comparing the differnet experimental groups**.

Slices from large intetsine of mice were studied stain them with hematoxilin-eosin. Microphotographs B, C, and D were taking to compare the mice only fed with yoghrut, others treated with DMH and fed with yoghrut and the tumour control group. All the samples were obtained in the end of the study (6 months).

A) Non-treatment control group (100X)
B) Yoghurt group. Tissue maintaine the typical structure of the large intestine but it is possible observe cell increase in the lamina propria (100X).
C) Yoghurt-DMH-yoghurt group. The tissue organization is conserve with inportant increases in the lamina propria cells and goblet cells (100X).
D) DMH group. Tumor tissue compound of fibrous tissue and queratin nucleus (arrow) (100X).

Figure 2. **Comparative study of two regulatory cytokines.**

Cytokines were analized in large intetsine slices from different experimental groups using indirect immunofluorescent technique. Results are expresed as number of positive cells counted in ten fileds of vision at 1000X of magnification. * represent significative diference comparing with the non-treatment control using Student's Test P<0,001.

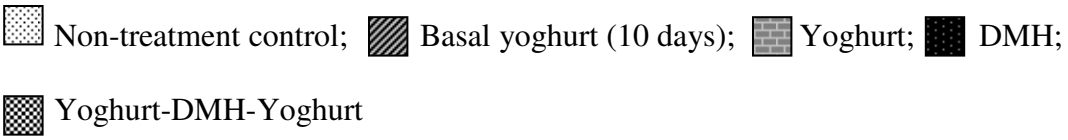

***Figure 3*. Interaction of yoghurt bacteria antigens with the small and large intestine of the mice.**

Fluorescent cells were observed on histological sections from small and large intestine of the mice, 30 or 60 min after intragastric administration of yoghurt bacteria labelled with FITC.

A) Fluorescent cells in lamina propria of the small intestine at 30 min.

B) Fluorescence observed in Peyers's patch at 30 min.

C) Fluorescent cells in the lamina propria of the large intestine at 30 min.

D) Bacteria interaction with large intestine cells at 60 min.

E) Low fluorescence in the large intestine nodule at 60 min.