Kefir is a fermented milk (drink) produced by the action of lactic acid bacteria, yeasts and acetic acid bacteria by studying their modulatory activity on the intestinal immune response. A functional dose was established in a murine model and the pattern of regulatory and pro-inflammatory cytokines induced was also studied. The existence of a common mucosal immune system implies that the immune cells stimulated in one mucosal tissue can spread and relocate through various mucosal sites. The aim of this work was to determine the effect of an oral administration of kefir on the duration of the intestinal mucosa immune response and the modulatory activity in distal mucosal sites, specifically in the peritoneal and pulmonary macrophages and in the bronchial tissue. BALB/c mice were fed with kefir or pasteurized kefir at doses previously determined as functional for intestinal mucosa immunomodulation. Kefir feeding was stopped and the number of IgA, IgG, IL-4, IL-6, IL-10, IFNγ and TNFα producing cells was determined in the lamina propria of small intestine immediately, and after 2 and 7 days of kefir withdrawal. IgA producing cells were also measured in the bronchial tissue of lungs immediately and 2 and 7 days after kefir withdrawal. Phagocytic activity of peritoneal and pulmonary macrophages was also determined. The oral administration of kefir or pasteurized kefir increased the number of IgA+ cells not only in the gut lamina propria, but also in the bronchial tissue, supporting the concept of local antibody secretion after remote-site stimulation in the intestinal tract. Both peritoneal and pulmonary macrophages were activated by kefir or pasteurized kefir feeding. Peritoneal macrophages were stimulated faster than pulmonary macrophages (for kefir). The enhanced phagocytic activity achieved by kefir or pasteurized kefir lasted longer for the peritoneal than for the pulmonary macrophages. Due to the increased bronchial IgA and phagocytic activity of pulmonary macrophages after kefir feeding observed in this study, the oral administration of kefir could act as a natural adjuvant for enhancing the specific immune response against respiratory pathogens. The parameters studied returned to control values within a week of cessation of kefir administration. This would suggest that there is a low risk of overstimulating the gut mucosal immune system during periodic consumption of viable kefir.

The oral administration of fermented dairy products has been shown to strengthen the nonspecific immune response or to act as an adjuvant in the antigen-specific immune response (1-2), mainly in the intestinal environment. Fermented milk administered to mice resulted in significant effects on vari-

Key words: immunomodulation, kefir, intestinal mucosa, distal sites.
ous immune responses including increased IgA-producing cells in a dose-dependent way, (3-4), increased macrophage activity (5), increased specific antibody responses during infections (6-7) and prevention of certain types of cancer (8-10).

Kefir is a fermented milk drink produced by the action of lactic acid bacteria, yeasts and acetic acid bacteria, trapped in a complex matrix of polysaccharides and proteins. As well as its inherent high nutritional value as a source of proteins and calcium, kefir has a long tradition of being regarded as good for health in countries where it is a staple part of the diet. However, human or animal nutritional surveys that have been published to substantiate this view, are not numerous (11). Studies have been published concerning non-immune (12-17) and immune system-related (18-21) effects of kefir. In a recent work (22) we performed a comparative study on the effect of kefir containing viable or non-viable bacteria by studying its modulatory activity on the intestinal immune response. A functional dose was established in a murine model, and the patterns of regulatory and pro-inflammatory cytokines induced were also studied.

The mucosal associated lymphoid tissue (MALT) constitutes a complex system of defense mechanisms that include all the immune cells associated with the gut (GALT), bronchus (BALT) and nasal (NASAL) tissues and other mucosa, such as genital, salivary and mammary glands (3). When the mucosal immune response is induced, primed T and B cells migrate through the lymphatic system and then enter the peripheral blood circulation via the thoracic duct. Extravasation of the immune cells occurs not only in the gut lamina propria but also in other mucosal sites such as the respiratory, urogenital or mammary tissues or salivary glands. This migration is known as the IgA cycle (23). A common mucosal immune system exists whereby immune cells stimulated in one mucosal tissue can spread and relocate to various mucosal sites (3). This concept implies that oral immune stimulation can induce immunity in distal intestinal mucosal sites. For instance, the consumption of fermented milk has been related to a reduced incidence of nasal pathogens (24-26). The majority of research concerning probiotic-mediated enhanced immune protection has focused on the gastrointestinal tract, and few studies have been conducted to consider the possibility that probiotics might stimulate the common mucosal immune system sufficiently to provide increased protection to other mucosal sites as well (27). The aim of this work was to determine the effect of oral administration of kefir on the duration of the intestinal mucosa immune response, and the modulatory activity in distal mucosal sites, specifically in the peritoneal and pulmonary macrophages and in the bronchial tissue of mice.

**MATERIALS AND METHODS**

**Kefir Samples**

Unflavoured kefir was produced by Les Produits de Marque Liberté (Candiac, Québec, Canada) from pasteurized bovine milk containing 1.8% fat. Kefir was obtained immediately after production and rapidly cooled in ice water or pasteurized. Samples (1L) of kefir to be pasteurized were poured aseptically into a 2L glass Erlenmeyer flask, heated at 62.5 °C for 30 min and then rapidly cooled in ice water. All samples were sealed and packed and were kept on ice until use. Microbiological analyses of the pasteurized products were performed to verify the effectiveness of the heat treatment.

**Animals and Feeding Procedures**

6 - 8 week-old BALB/c female mice weighing 20 to 25 g were obtained from the Charles River Company (Montreal, Canada). Each experimental group (sample) consisted of 5 mice housed together in a plastic cage kept in a controlled atmosphere (temperature 22 ± 2 °C; humidity 55 ± 2%) with a 12 h light/dark cycle. Mice were maintained and treated in accordance with the guidelines of the Canadian Council on Animal Care.

Animals were offered kefir or pasteurized kefir at the conditions recently determined as functional (Vinderola et al. 2004). Kefir was offered diluted 1/100 and *ad libitum* for 2 consecutive days, whereas pasteurized kefir was diluted 1/10 and offered for 5 consecutive days (as a replacement for water) (22). Kefir dilutions were done in Phosphate Buffered Saline (PBS) solution (Sigma-Aldrich, St. Louis, MO, U.S.A.). The daily kefir intake was 3.1 ± 0.3 ml/day/mouse. All groups of mice received simultaneously a conventional balanced diet *ad libitum*. A control group received the same conventional balanced diet, but with water instead of kefir. To evaluate the duration of the immunostimulating effects, mice were fed as described above. After these feeding periods (2d for kefir and 5d for pasteurized kefir), kefir or pasteurized kefir was removed from the cages and animals were fed *ad libitum* the conventional balanced diet and water. At the end of each feeding period with kefir (2d 1/100) or pasteur-
ized kefir (5d 1/10) and after 2 or 7 days of kefir or pasteurized kefir withdrawal, animals were anesthetized and sacrificed by cervical dislocation to obtain the different tissues for the immunological studies.

**Ex vivo Phagocytosis Assay of Peritoneal Macrophages**

The assay was performed according to Perdigón et al. (5). Briefly, peritoneal macrophages were harvested by washing the peritoneal cavity with 5 ml of PBS containing 10 U mL\(^{-1}\) of heparin (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 0.1% bovine serum albumin (Jackson ImmunoResearch, West Grove, PA, USA). The macrophage suspension was washed twice with the same buffer, and it was adjusted to a concentration of 10⁶ cells mL\(^{-1}\). A heat-killed (100ºC, 15 min) *Candida albicans* suspension (10⁷ cells mL\(^{-1}\)) was opsonized with mouse autologous serum (10%) for 15 min at 37ºC. 0.2 mL of the opsonized yeasts was added to 0.2 mL of each macrophage suspension. The mixture was incubated for 30 min at 37ºC. The percentage of phagocytosis was measured as the % of activated (with at least one yeast cell phagocyted) macrophages after a 100-cell count using an optical microscope.

**Ex vivo phagocytosis assay in pulmonary macrophages**

Lungs were minced with scissors into 1 mm² fragments and forced to pass through a metal mesh. Lung fragments were collected in 20 ml of PBS containing 10 U mL\(^{-1}\) of Heparin (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 0.1% bovine serum albumin (Jackson ImmunoResearch, West Grove, PA, USA). The lung suspension was incubated (45 min, 100 rpm, room temperature) in the presence of 300 U mL\(^{-1}\) of collagenase (C-7657 Sigma-Aldrich, St. Louis, MO, U.S.A.) and 0.1 mg mL\(^{-1}\) of dispase (Gibco, Invitrogen, Burlington, ON, Canada). The pulmonary macrophage suspension was allowed to stand for 2 min and the supernatant was centrifuged (2000 rpm, 10 min 4°C) and washed twice with Dulbecco’s Modified Eagle’s Medium (DMEM) (ATCC, Manassas, VA, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (ATCC) and 10 U/ml of heparin (Sigma-Aldrich). The pellet was resuspended in 4 ml of the same culture medium and placed in Leighton tubes (1 mL) containing a 50x10 mm microscope cover glass, for macrophage adhesion (37°C, 1 h, in a 5% CO₂ incubator). After the adhesion period, cover glasses were gently washed twice with the culture medium and 2 mL of an opsonized heat-inactivated *Candida albicans* suspension (10⁶ cells mL\(^{-1}\)) was added. Leighton tubes were then incubated for 30 min (37°C, in a 5% CO₂ incubator). The percentage of phagocytosis was measured as the % of activated macrophages after a 100-cell count using an optical microscope.

**Cytokine production by cultured peritoneal and pulmonary macrophages**

The peritoneal or pulmonary suspensions of macrophages obtained as described above were placed in Leighton tubes (1 mL) and incubated (37°C, 1 h, in a 5% CO₂ incubator) to allow the adhesion of macrophages to the cover glass. Then, DMEM containing 10% FBS (ATCC), 100 U mL\(^{-1}\) of penicilin and 0.1 mg mL\(^{-1}\) streptomycin were added. Macrophages were incubated for 72 h and the supernatant was recovered and kept frozen until cytokine analyses. IL-1α, IL-1β and IFNγ were determined on the supernatants of the macrophage culture using the corresponding mouse IL-1α, IL-1β or IFNγ ELISA Set (BD OptEIA, BD Biosciences PharMingen, San Diego, CA, USA).

**Immunofluorescence test for B population (IgA⁺ and IgG⁺ cells) identification**

The small intestine and the lung were removed for histological preparation following Sainte-Marie technique (28) for paraffin inclusion. The number of IgA producing (IgA⁺) cells was determined on histological slices of samples from the ileum region near Peyer’s patches and on bronchial tissue by a direct immunofluorescence method (29). The number of IgG⁺ cells was also determined on histological slices of the small intestine. The immunofluorescence test was performed using (a-chain specific) anti-mouse IgA FITC conjugate or (g-chain specific) anti-mouse IgG FITC conjugate (Sigma-Aldrich, St. Louis, MO, U.S.A.). Histological slices were deparaffinized and rehydrated in a graded series of ethanol. Deparaffinized histological samples were incubated with the appropriate antibody dilution (1/100 for IgA or 1/50 for IgG) in PBS solution for 30 min at 37°C. Then, samples were washed 2 times with PBS solution and examined using a fluorescent light microscope. The results were expressed as the number of IgA⁺ or IgG⁺ cells (positive: fluorescent cell) per 10 fields (magnification 100x). Data represent the mean of three histological slices for each animal, for each sampling point.

**Cytokine assays**

IL-4, IL-6, IL-10, IFNγ and TNFα were studied by an indirect immunofluorescence method. Small intestine was removed and processed for histological preparation as described above. Histological slices were deparaffinized and rehydrated in a graded series of ethanol, and then incubated for 30 min in a 1% blocking solution of BSA
Histological slices were then incubated for 60 min at 37°C with rabbit anti-mouse IL-4, IL-6, IL-10, IFNg or TNFa (Peprotech, Inc., Rocky Hill, NJ, U.S.A) polyclonal antibodies. The incubation was followed by 2 washes with PBS solution. Histological slices were treated for 45 min at 37°C with a dilution of a goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research), washed twice with PBS and examined using a fluorescent light microscope. The results were expressed as the number of IL-4+, IL-6+, IL-10+, IFNg+ or TNFa+ cells (positive: fluorescent cell) per 10 fields (magnification 100x). Data represent the mean of three histological slices for each animal, for each sampling point.

Statistical analysis
Data were analyzed using the one-way ANOVA procedure of SPSS software. The differences among means were detected by the Duncan’s Multiple Range Test (30). Data were considered significantly different when $p < 0.05$.

RESULTS

Analysis of the B population by the determination of IgA and IgG producing cells

The number of IgA+ cells on histological slices of small intestine and bronchial tissues after the oral administration of kefir and pasteurized kefir is shown in Figure 1. A significant increase in the number of IgA+ cells was detected in both tissues immediately after the oral administration of kefir (2d 1/100) or pasteurized kefir (5d 1/10). The immune response observed in the bronchial and intestinal tissues steadily decreased after the cessation of kefir feeding. The number of IgA+ cells returned to control values in the bronchial and intestinal tissues. No significant increase of IgG+ cells in the intestinal tissue was observed after kefir feeding. However, a significant increase in this cell population was observed for pasteurized kefir feeding, which returned to normal values 7 days after pasteurized kefir withdrawal (Fig. 2).

Phagocytosis of peritoneal and pulmonary macrophages

The phagocytic activities of peritoneal and pulmonary macrophages after kefir and pasteurized kefir administration are shown in Figure 3. A significant increase of phagocytosis in the peritoneal macrophages was observed for both kefir and pasteurized kefir feeding, and this enhanced phagocytosis was maintained even 7 days after kefir withdrawal. An increased phagocytic activity in the pulmonary macrophages was observed only in those mice that had received pasteurized kefir for 5d (Fig. 3 B). This enhanced phagocytosis steadily decreased after the cessation of pasteurized kefir feeding but still after a week it was higher than control values. In

![Graph A](image1.png)

**Fig. 1 -** Effect of the oral administration of kefir 1/100 for 2d (K 1/100) and pasteurized kefir 1/10 for 5d (PK 1/10) on the number of IgA+ cells on histological slices of small intestine (A) and bronchial tissue (B) immediately after (0 d, _) each feeding period and after 2 (_ ) and 7d (_) of kefir or pasteurized kefir withdrawal. * Significantly different from control ($p < 0.05$).
order to determine whether the 2d feeding period with kefir diluted 1/100 had been not enough to activate pulmonary macrophages, additional mice were fed for 2 and 5d with both kefir (1/100) and pasteurized kefir (1/10). We observed that a feeding period of 5d was necessary for kefir diluted 1/100 or pasteurized kefir diluted 1/10 to enhance the phagocytosis in pulmonary macrophages (Fig. 4).

**Cytokine analyses**

A significant increase, compared to control mice, in the production of IL-1α by cultured peritoneal macrophages was observed immediately after kefir and pasteurized kefir feeding (Fig. 5). It returned to control values as soon as kefir was withdrawn from the diet. No differences in the content of IL-1α in the supernatant of the culture of pulmonary macrophages were detected, compared to controls (data not shown). No IL-1β or IFNγ were detected in the supernatant of peritoneal or pulmonary macrophages of control or treated animals.

The number of IL-4+, IL-6+ and IL-10+ cells increased in the small intestine of mice that received kefir or pasteurized kefir (Figs. 6A, B and C). 7 days after kefir withdrawal all values returned to control ones, following the same pattern as IgA. A slight but significant increase in the number of IFNγ+ cells was observed in the small intestines of mice that received kefir for 2d. The peak for IFNγ production in mice eating pasteurized kefir was observed 2d after its withdrawal (Fig. 5D). TNFα did not increase in mice that received kefir, and in those that received pasteurized kefir, the increase observed returned to control values within a week of pasteurized kefir withdrawal (Fig. 5E).

**DISCUSSION**

The immunopotentiating activity of fermented dairy products and their impact on the intestinal
humoral response are known (31,32,33). Previous reports showed that kefir was able to enhance the specific intestinal mucosal immune response against cholera toxin (19). The immunodulating activity of kefir or kefiran (a polysaccharide found in kefir) in tumor bearing mice has been reported (18,20). In a previous work, we studied the influence of the dose and kefir bacterial cell viability on the capacity of kefir to increase the number of lamina propria IgA+ cells. Kefir containing viable bacteria was able to achieve a similar effect on the number of IgA+ cells as did pasteurized kefir, even though the former had been administered 10 times less concentrated than the latter. According to the number of IgA+ and IgG+ cells in the small intestine lamina propria and to haematoxilin-eosin studies, 2 days of feeding with kefir diluted 1/100 and 5 days of feeding with pasteurized kefir diluted 1/10 were the conditions able to best modulate the intestinal mucosal immune response (22).

The main function of secretory IgA in the gut is to exert immune exclusion by intimate cooperation with the innate non-specific defence mechanisms. In this work, we observed that the oral administration of kefir or pasteurized kefir increased the number of IgA+ cells not only in the gut lamina propria, but also in the bronchial tissue, supporting the concept of local antibody secretion after remote-site stimulation in the intestinal tract. Previous reports demonstrated that oral administration of certain strains of lactic acid bacteria (3) or fermented milks (34) induced an IgA cycle and an increase of this cell population at the bronchial level. An immunological communication exists between intestinal and pulmonary mucosa. This connection between mucosal surfaces allows immunity initiated at one anatomical site to protect another mucosal site (35). In both cases, IgA+ cells returned to normal values in a 2-7 days period after the cessation of the stimulation with kefir, which is in accordance with the 5 days of half-life for the majority of IgA plasma cells (36). IgG should not be considered as an important immunoglobulin at the intestinal mucosal because its external translocation depends on passive intercellular diffusion and increases during intestinal inflammatory processes (37,38). In this study, a significant increase in the number of IgG+ cells was observed in mice consuming pasteurized kefir. This increase

**Fig. 4** - Effect of the oral administration of kefir 1/100 (K 1/100) and pasteurized kefir 1/10 for 2 (·) and 5d (△) on the percentage of phagocytosis of pulmonary macrophages. * Significantly different from control (p < 0.05).

**Fig. 5** - Effect of the oral administration of kefir 1/100 for 2d (K 1/100) and pasteurized kefir 1/10 for 5d (PK 1/10) on the IL-1α production by peritoneal macrophages immediately after (0 d, _) each feeding period and after 2 (·) and 7d (△) of kefir or pasteurized kefir withdrawal. * Significantly different from control (p < 0.05).
in intestinal IgG had no detrimental effects in the gut, since no tissue damage or alteration of intestinal structures had been observed, as revealed by previous haematoxilin-eosin studies (22). Macrophages are considered the first line of defense in the immune response to foreign invaders. Oral administration of viable probiotic bacteria represents a way to stimulate the host’s non-specific immunity by enhancing the systemic immune response or by modulating the functions of immunocompetent cells (5). In this study, we used peritoneal cavity lavage to harvest peritoneal macrophages. However, for pulmonary macrophages, we dissociated lung tissue using a mechanical and enzymatic technique since it was reported that while bronchoalveolar lavage is very useful to harvest interstitial macrophages, it removes alveolar macrophages only in part despite extensive lavage (39). In this work, both peritoneal and pulmonary macrophages were activated by kefir or pasteurized kefir feeding. Peritoneal macrophages were stimulated faster than pulmonary macrophages (for kefir) and the enhanced phagocytic activity achieved by kefir or pasteurized kefir lasted longer for peritoneal than for pulmonary macrophages. This is may be due to the fact that the former are located anatomically closer to the place (intestinal environment) where the macrophage stimulating cytokines are produced. Yasui et al. (40) reported a murine model in which oral administration of Bifidobacterium breve followed by oral immunization with influenza virus protected against viral infection of the lower respiratory tract. Alvarez et al. (41) also found that orally administered Lactobacilli casei and yogurt prevented Pseudomonas aeruginosa infection in young mice. Due to the increased bronchial IgA and phagocytic activity of pulmonary macrophages observed in this study followed kefir feeding, the oral administration of kefir could act as a natural adjuvant for enhancing the specific immune response against respiratory pathogens. Whether the increase in phagocytic capacity is maintained after cessation of lactic acid bacteria (LAB) feeding is not clear (42). Schiffrin et al. (43) noted that the increased phagocytic activity in human subjects persisted for 6 weeks after ingestion of LAB. In accordance with this observation, we were able to observe increased phagocytosis in peritoneal and pulmonary macrophages after pasteurized kefir withdrawal (e.g. 7 days).

B cell immunoglobulin switching and differentiation to plasmocytes secreting IgA occurs upon interactions with T cells in the lamina propria in an environment rich in IL-4, IL-5, IL-10 and TGF-beta (44). IL-6 promotes terminal differentiation of B cells into plasma cells (45). In this study, IL-4, IL-6 and IL-10 significantly increased in the lamina propria of the small intestine of animals that received both types of kefir, which is in accordance with the increase in the number of IgA+ cells observed. It has been reported that IL-6 is responsible for the shifting of the Th1/Th2 balance towards the Th2 direction using two independent approaches: promoting IL-4 production and Th2 differentiation and inhibiting IFNg production and Th1 differentiation (46). This phenomenon was observed in this study, where the magnitude of the increase for Th2 cytokines (IL-4 and IL-10) was much more evident than for Th1 cytokines (IFNg and TNFa). It has been also stated that IL-10 and IL-6 synergize to sustain Ig secretion (47).

The maximum in IFNg and TNFa production was
not found on the same day after pasteurized kefir administration. This difference in the kinetics of the expression of cytokines was also observed when different *Lactobacillus* were administered to mice (48). Macrophage function has long been known to be controlled by activated T cells. IFNg and TNFa are involved in macrophage activation (49,50). An increased phagocytic activity of peritoneal macrophages, but low production of IFNg, was observed after 2d of kefir administration. This increased phagocytic activity could be due to an alternative activation state of macrophages that is distinct from the classical Th1 type activation by IFNg, that would be mediated by Th2 cytokines IL-4 and IL-13 (51).

Orally administered lactobacilli can activate macrophages, with subsequent production of TNFa and IL-1 (48). In this study, IL-1a was found in the supernatant of cultured macrophages isolated from the peritoneal cavity of mice that received kefir or pasteurized kefir (the latter contained a greater load of (dead) bacterial cells), but in significant different quantities. These *in vivo* findings confirm previous *in vitro* findings (45) that demonstrated the existence of thresholds of bacterial concentration necessary to induce cytokine production. At the same time, our findings agree with those of Haller et al. (52), who determined that the amount of bacteria necessary to induce maximal TNFa secretion was higher for heat-killed bacteria than for live bacterial cells. Some studies of the immunomodulatory properties of dietary LAB have drawn the general conclusion that live bacteria are more effective for modulating immunity than killed bacteria (53). Our results are in accordance with this statement, since while kefir was administered 10 times more diluted than pasteurized kefir, it induced an immunostimulation of similar magnitude to the latter. All the experimental results presented here suggest that a Th1 response was controlled by Th2 cytokines for mice consuming kefir. In mice consuming pasteurized kefir, a Th2 profile was observed and a significant Th1 response also took place. It was established that the benefits of probiotics were found to be greater the more advanced the age and/or the more impaired the immune parameters at baseline: both Th1 and Th2 cytokine patterns could be partially restored (54). A recent work (55) showed that enhanced intestinal IgA levels could be maintained during long-term administration of yogurt. It was also reported that chronic yogurt consumption in humans led to no adverse effects on immunity but lowered the level of allergies for the group consuming live yogurt (56). The immune parameters studied here returned to control values within a week of cessation of kefir consumption. This suggests that periodic consumption of viable kefir is safe and does not pose a risk of overstimulating the gut mucosal immune system.

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