

## Immunomodulating capacity of kefir

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Kefir is a fermented milk produced by the action of lactic acid bacteria, yeasts and acetic acid bacteria, trapped in a complex matrix of polysaccharides and proteins. Beyond 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 in the diet. However, published human or animal feeding trials to substantiate this view are not numerous. The aim of this work was to determine the immunomodulating capacity of kefir on the intestinal mucosal immune response in mice and to demonstrate the importance of dose and cell viability on this response. BALB/c mice were fed with commercial kefir *ad libitum* (diluted 1/10, 1/50, 1/100 or 1/200) or pasteurized kefir (diluted 1/6, 1/10, 1/50, 1/100) for 2, 5 or 7 consecutive days. At the end of each feeding period, the bacterial translocation assay was performed in the liver. Small intestine structure was studied by haematoxylin-eosin staining and light microscopy. The number of IgA+ and IgG+ cells was also determined. For the functional doses chosen, cytokines (IL-2, IL-4, IL-6, IL-10, IL-12, TNF- $\alpha$  and IFN- $\gamma$ ) were determined. Kefir and pasteurized kefir were able to modulate the mucosal immune system in a dose-dependent manner. Kefir was administered 10-times more diluted than pasteurized kefir, but it induced an immunomodulation of similar magnitude, indicating the importance of cell viability. The results suggest that a Th1 response was controlled by Th2 cytokines induced by kefir feeding. Pasteurized kefir would induce both Th2 and Th1 responses. This is the first study *in vivo* regarding the mechanisms involved in the immunomodulating capacity of the oral administration of kefir containing viable or heat-inactivated bacteria at different doses.

**Keywords:** Probiotics, fermented milks, mucosal immunity.

Fermented dairy products have long played an important role in the diet of people around the world. They produce a wide range of physiological and therapeutic effects, including stimulation of the immune system (Gill, 1998). Numerous studies have demonstrated the beneficial effects of lactic acid bacteria in boosting specific or nonspecific immune responses (Isolauri et al. 2001), the importance of dose (Perdigón et al. 1991; Marin et al. 1998; Vinderola et al. 2004) and cell-viability (Ouweland & Salminen, 1998; Haller et al. 1999; Gill & Rutherford, 2001; Galdeano & Perdigón, 2004). Probiotic microorganisms can exert their beneficial properties through two mechanisms: direct effects of the live microbial cells (probiotics) or indirect effects via metabolites of these cells (biogenics). Biogenics

are defined as food components derived from microbial activity which provide health benefits without involving the intestinal microflora (Takano, 2002). The most important biogenics in fermented milk may be peptides that are not present prior to fermentation. It is generally assumed that, to exert positive health effects, probiotic microorganisms in a dairy product need to be viable. However, the use of non-viable instead of viable microorganisms would be economically attractive because of longer shelf-life and reduced requirements for refrigerated storage. Additionally, fermented-then-pasteurized products could expand the potential use of probiotics to areas where strict handling conditions cannot be met, e.g., developing countries (Ouweland & Salminen, 1998).

Fermented milks administered to mice resulted in significant effects on various immune responses such as increased IgA-producing cells in a dose-dependent way,

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(Perdigón et al. 1999, 2001), increased macrophage activity (Perdigón et al. 1986), increased specific antibody responses during infections (Perdigón et al. 1991; Cano & Perdigón, 2003) and prevention of certain types of cancer (Perdigón et al. 2002; Rachid et al. 2002; de Moreno de LeBlanc & Perdigón, 2004). Matar et al. (2001) and LeBlanc et al. (2002) first described the immunomodulating capacity *in vivo* of peptides released during milk fermentation on the intestinal mucosal immune system.

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. Beyond its inherent high nutritional value as a source of protein and calcium, kefir has a long tradition of being regarded as good for health in countries where it is a staple in the diet. However, published human or animal feeding trials to substantiate this view are not numerous. Although the Russian literature contains articles describing the effects of kefir consumption, details of these studies are not always readily available in western countries (Farnworth, 1999, Farnworth & Mainville, 2003). Consumption of this fermented milk has been shown to enhance the intestinal hydrolysis of lactose in pigs (de Vrese et al. 1992) and to improve lactose digestion and tolerance in adults (Hertzler & Clancy, 2003). Oral administration of milk and soy milk kefirs (Liu et al. 2002) or kefiran (Murofushi et al. 1986) – an exopolysaccharide produced by bacteria found in kefir – significantly inhibited the growth of tumours in mice inoculated with sarcoma 180 tumour cells. Thoreux et al. (2001) showed that orally administered kefir enhanced the specific mucosal immune response against cholera holotoxin in young adult, but not in senescent rats. It is not clear yet whether kefir's health-promoting effects are due to one particular bacterium or yeast (Yuksekdag et al. 2004), to kefiran or to peptides released during milk fermentation or whether it is a synergic combination of these factors. Information on the pattern of cytokines induced in the intestinal environment following the oral intake of kefir would greatly contribute to the elucidation of the mechanisms involved in its modulation of the immune response. The aim of the present work was to determine the immunomodulating capacity of kefir on the intestinal mucosal immune response in mice and to ascertain the importance of dose and cell viability on this response.

## 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 (1 l) of kefir to be pasteurized were poured aseptically into a 2-l 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*

BALB/c female mice, 6–8 weeks old and weighing 20–25 g, were obtained from Charles River (Montreal, Canada). Each experimental group consisted of five mice housed in plastic cages 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 *ad libitum* (diluted 1/10, 1/50, 1/100 or 1/200) or pasteurized kefir (diluted 1/6, 1/10, 1/50, 1/100) (substituted for drinking water) for 2, 5 or 7 consecutive days. Daily intake of kefir was 3.1±0.3 ml/mouse. Kefir dilutions were done in phosphate-buffered saline (PBS) solution (Sigma-Aldrich, St. Louis MO, USA). All groups of mice (including control mice) received simultaneously a conventional balanced diet *ad libitum*. The control group received the same conventional balanced diet, but water instead of kefir. Animals were fed in a way that all groups (2, 5, 7 d and control) were killed on the same day.

### *Bacterial translocation assay*

At the end of each feeding period, mice were anaesthetized and killed by cervical dislocation and their livers removed aseptically. Organs were homogenized in 5 ml 0.1% sterile peptone water and 1 ml of each liver homogenate was plated on MacConkey agar (for enterobacteria) and LAPTg agar (for other facultative anaerobic microorganisms). Plates were incubated under aerobic conditions for 48 h at 37 °C. At the end of the incubation period, plates were examined and the result was expressed as positive (presence of bacteria on plates) or negative (absence of bacteria on plates) bacterial translocation. Translocation was considered to have occurred when colonies were observed on agar plates, since the liver is an organ normally devoid of bacteria.

### *Histological studies of the gut*

Small intestine was removed from treated and control animals after each feeding period. Intestines were processed for paraffin inclusion following the Sainte-Marie technique (Sainte-Marie, 1962). Serial paraffin-sections (4 µm) were stained with haematoxylin-eosin followed by light microscopy examination.

### *Immunofluorescence test for B population (IgA<sup>+</sup> and IgG cells) identification*

The number of IgA-producing cells was determined on histological slices of samples from the ileal region near

**Table 1.** Effect of the oral administration of kefir and pasteurized kefir at different dilutions on the number of IgA+ and IgG+ cells on histological slices of small intestine of miceValues are the mean  $\pm$  SD of 5 animals/treatment/d

Days of feeding	N° IgA+ cells/10 fields					
	Kefir dilution		Pasteurized kefir dilution			
	1/00	1/200	1/6	1/10	1/50	1/100
Control	81.2 $\pm$ 3.6 <sup>a</sup>					
2 d	99.5 $\pm$ 3.4 <sup>b</sup>	86.7 $\pm$ 3.5 <sup>a</sup>	104.2 $\pm$ 6.0 <sup>b</sup>	102.7 $\pm$ 4.5 <sup>b</sup>	91.3 $\pm$ 3.4 <sup>b</sup>	92.7 $\pm$ 5.5 <sup>b</sup>
5 d	96.3 $\pm$ 5.9 <sup>b</sup>	87.6 $\pm$ 1.7 <sup>a</sup>	114.2 $\pm$ 2.7 <sup>b</sup>	107.1 $\pm$ 3.6 <sup>b</sup>	94.8 $\pm$ 5.0 <sup>b</sup>	88.1 $\pm$ 2.8 <sup>a</sup>
7 d	85.9 $\pm$ 2.3 <sup>a</sup>	88.5 $\pm$ 2.7 <sup>a</sup>	85.2 $\pm$ 2.9 <sup>a</sup>	78.6 $\pm$ 2.5 <sup>a</sup>	92.3 $\pm$ 4.2 <sup>b</sup>	87.9 $\pm$ 2.8 <sup>a</sup>

Days of feeding	N° IgG+ cells/10 fields					
	Kefir dilution		Pasteurized kefir dilution			
	1/100	1/200	1/6	1/10	1/50	1/100
Control	35.0 $\pm$ 2.8 <sup>a</sup>					
2 d	38.6 $\pm$ 2.4 <sup>a</sup>	35.6 $\pm$ 2.1 <sup>a</sup>	55.8 $\pm$ 2.6 <sup>b</sup>	42.5 $\pm$ 3.3 <sup>b</sup>	37.2 $\pm$ 1.7 <sup>a</sup>	34.8 $\pm$ 3.2 <sup>a</sup>
5 d	37.9 $\pm$ 3.0 <sup>a</sup>	33.4 $\pm$ 3.0 <sup>a</sup>	42.7 $\pm$ 2.7 <sup>b</sup>	45.5 $\pm$ 3.7 <sup>b</sup>	36.2 $\pm$ 2.2 <sup>a</sup>	33.0 $\pm$ 4.4 <sup>a</sup>
7 d	39.3 $\pm$ 3.2 <sup>a</sup>	38.0 $\pm$ 2.9 <sup>a</sup>	63.2 $\pm$ 3.7 <sup>b</sup>	46.0 $\pm$ 1.6 <sup>b</sup>	35.8 $\pm$ 2.2 <sup>a</sup>	35.6 $\pm$ 2.3 <sup>a</sup>

<sup>a,b,c,d</sup> Values in the same column without a common superscript are significantly different from the corresponding control value ( $P < 0.05$ )

Peyer's patches by direct immunofluorescence (Vintiñi et al. 2000). Small intestine was removed for histological preparation as described above. The immunofluorescence test was performed using ( $\alpha$ -chain specific) anti-mouse IgA FITC conjugate or ( $\gamma$ -chain specific) anti-mouse IgG FITC conjugate (Sigma-Aldrich). 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. Samples were then washed three times with PBS solution and examined using a fluorescent light microscope. Results were expressed as the number of IgA- or IgG-producing cells (positive: fluorescent cell) per 10 fields (magnification 100 $\times$ ). Results are means of three histological slices for each animal, for each feeding period.

#### Cytokine assay

At the end of each feeding period, the small intestine was removed and processed for histological preparation as described above. IL-2, IL-4, IL-6, IL-10, IL-12, TNF- $\alpha$  and IFN- $\gamma$  were studied by indirect immunofluorescence. 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 (Jackson Immuno Research, West Grove PA, USA) at room temperature. Histological slices were then incubated for 60 min at 37 °C with rabbit anti-mouse IL-2, IL-4, IL-6, IL-10, IL-12, TNF- $\alpha$  or IFN- $\gamma$  (Peprotech Inc., Rocky Hill NJ, USA) polyclonal antibodies. The incubation was followed by two washes with PBS solution and, finally, sections were treated for 45 min at 37 °C with a dilution of a goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research). Results

were expressed as the number of X-producing cells (positive: fluorescent cell) per 10 fields (magnification 100 $\times$ ). Results are means of three histological slices for each animal, for each feeding period.

#### Statistical analysis

Results for IgA- and IgG- and cytokine-producing cells were analysed using the one-way ANOVA procedure of SPSS software. Differences among means were detected by Duncan's Multiple Range Test (SPSS, 1996). Values were considered significantly different when  $P < 0.05$ .

## Results

#### Translocation assay

Enterobacteria were detected on MacConkey agar plates after the administration of kefir diluted 1/10 or 1/50 (results not shown). In view of these results, these dilutions of kefir were no longer considered in this study. For all the other dilutions of kefir or pasteurized kefir, no colonies were detected on either of the two agar media selected for the translocation assay.

#### 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 of mice fed with kefir or pasteurized kefir (which have not shown translocation to the liver) are reported in Table 1. All dilutions assessed showed a significant

**Table 2.** Effect of the oral administration of kefir (K, diluted 1/100) and pasteurized kefir (PK, diluted 1/10) on the number of IL-4+, IL-10+ and IL-6+ cells/10 fields on histological slices of small intestine of miceValues are the mean  $\pm$  SD of 5 animals/treatment/d

Days of feeding	IL-4		IL-10		IL-6	
	K 1/100	PK 1/10	K 1/100	PK 1/10	K 1/100	PK 1/10
Control	39.1 $\pm$ 1.1 <sup>a</sup>		35.4 $\pm$ 1.9 <sup>c</sup>		40.8 $\pm$ 2.6 <sup>e</sup>	
2 d	68.3 $\pm$ 2.2 <sup>b</sup>	74.5 $\pm$ 1.2 <sup>b</sup>	78.4 $\pm$ 2.7 <sup>d</sup>	69.4 $\pm$ 2.9 <sup>d</sup>	78.1 $\pm$ 3.4 <sup>f</sup>	85.1 $\pm$ 9.3 <sup>f</sup>
5 d	57.7 $\pm$ 2.3 <sup>b</sup>	100 $\pm$ 4.9 <sup>b</sup>	61.9 $\pm$ 2.5 <sup>d</sup>	77.1 $\pm$ 4.1 <sup>d</sup>	84.4 $\pm$ 3.7 <sup>f</sup>	105.9 $\pm$ 5.2 <sup>f</sup>
7 d	41.7 $\pm$ 3.2 <sup>a</sup>	84.8 $\pm$ 4.1 <sup>b</sup>	49.3 $\pm$ 4.3 <sup>d</sup>	85.7 $\pm$ 2.9 <sup>d</sup>	81.2 $\pm$ 4.3 <sup>f</sup>	79.7 $\pm$ 3.1 <sup>f</sup>

a,b,c,d,e,f Values in the same column without a common superscript are significantly different from the corresponding control value ( $P < 0.05$ )

increase in the number of IgA+ cells, with the exception of kefir diluted 1/200, and in a dose-dependent way. For pasteurized kefir, the highest values of IgA+ cells were observed after 5 d of feeding for the dilutions assayed. For kefir, the highest increase was observed after 2 and 5 d of feeding with the dilution 1/100. Table 2 also shows the number of IgG+ cells in animals fed under the same conditions described above. A significant increase in the value of this parameter was only observed for pasteurized kefir diluted 1/6 and 1/10. Figure 1 shows the morphological architecture (haematoxylin-eosin studies) of the small intestine of control mice compared with those animals that received kefir (diluted 1/100) or pasteurized kefir (diluted 1/10) for 2 or 5 consecutive days, respectively. No lymphocyte infiltrates and no oedema or mucosal atrophy were observed. No significant morphological changes in the overall architecture of the small intestine were observed when compared with control mice. According to these results, we chose the dilution 1/100 for kefir and 1/10 for pasteurized kefir as the functional dilutions for further study of cytokine profiles, since they were able to significantly increase the number of IgA producing cells at the intestinal mucosal level without causing an imbalance and translocation of the resident microflora or affecting the normal morphology of the small intestine.

#### Cytokine analyses

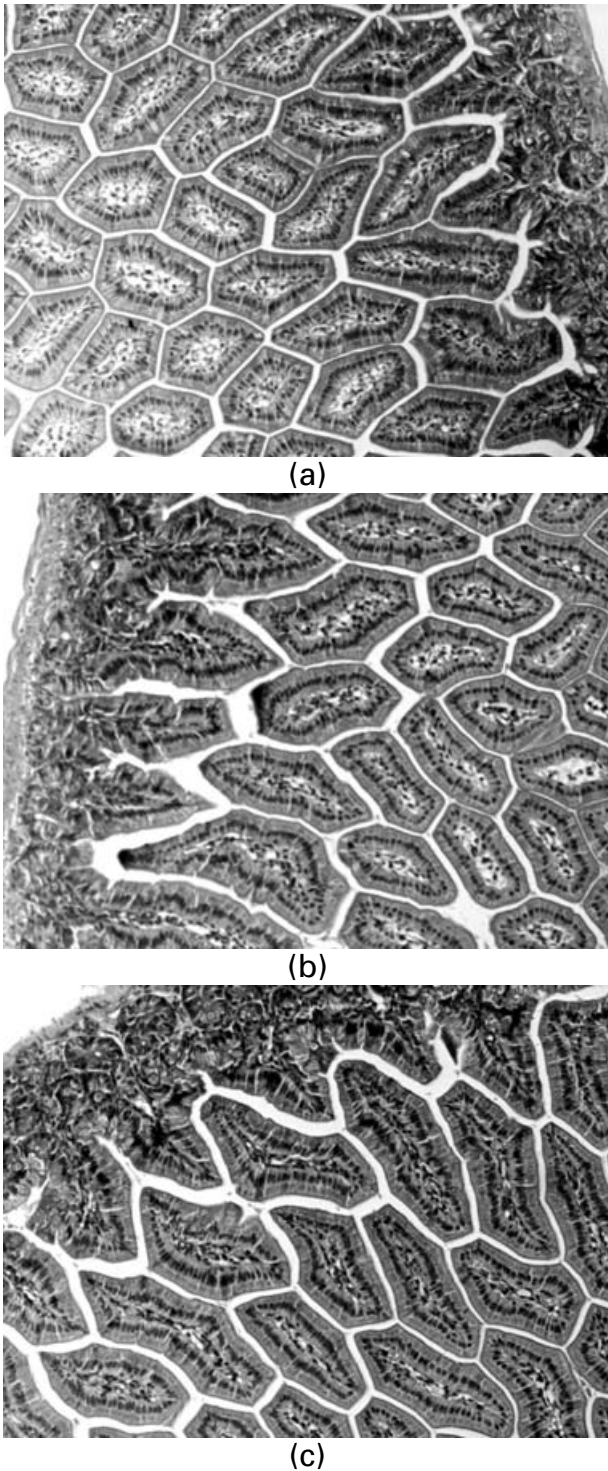
For the dilutions 1/100 and 1/10 of kefir and pasteurized kefir, respectively, a group of cytokines was studied by indirect immunofluorescence on the lamina propria of the small intestine of treated and control mice. Table 2 show the patterns observed for IL-4 and IL-10, respectively. IL-4+ cells significantly increased in both groups of mice fed with kefir (1/100) or pasteurized kefir (1/10), with a similar pattern of increase in the number of IgA+ cells. However, a constant increment in the number of IL-10+ cells was observed during the days assayed (2, 5 and 7 d) for pasteurized kefir, whereas for kefir, the most significant increase appears after 2 d of feeding. A significant increase in the number of IL-6+ cells (Table 2) in the small intestine of animals that received kefir or pasteurized kefir was also noticed. A moderate but significant increase in the number

of IL-2+ cells was observed for all the days assayed in animals fed with kefir (1/100) or pasteurized kefir (1/10) (Table 3). The number of IFN $\gamma$ + cells also increased (Table 3), except for the group of mice fed with kefir for 2 d. For TNF $\alpha$ + cells a significant increase was observed (Table 3) in mice that received pasteurized kefir and in the group that received kefir for 7 d. No significant increase was observed in the number of IL-12+ cells in the lamina propria of mice that received kefir. However, a significant increase of this pro-inflammatory cytokine was observed for all the days assayed in those mice that consumed pasteurized kefir (Table 3).

#### Discussion

The introduction of an intestinal immunomodulating food in the diet must be accompanied by the absence of side effects such as a strong inflammatory response (Perdigón et al. 2001). Among the possible adverse effects of regular probiotic feeding, the risk of bacterial translocation (passage of viable indigenous bacteria from the gut to the mesenteric lymph nodes and other sites beyond the intestine) should be evaluated carefully. The pharmacokinetic properties of a potential probiotic supplement should be studied to determine at which dose and for how long the product has to be administered to the host (Pavan et al. 2003) since it cannot be assumed that all probiotic organisms or products share the historical safety of tested or traditional strains. Animal studies, although of restricted value in microbiological risk assessment, are very important in determining dose-response effects (Salminen et al. 1998). In the present study, we performed the translocation assay in the liver of mice after the oral administration of different doses of kefir or pasteurized kefir in order to determine a functional dose, without producing translocation and causing an imbalance affecting the normal morphology of the small intestine. We determined that the dilutions 1/10 or 1/50 for kefir induced translocation of microflora to the liver. For the other dilutions assayed for kefir or pasteurized kefir, no translocation was observed.

The translocation of lactic acid bacteria from gut to extra-gut tissues, especially the mesenteric lymphoid node, was suggested as a normal and beneficial physiological



**Fig. 1.** Haematoxylin-eosin stain of histological slices of small intestine of control mice (a), mice that received kefir (diluted 1/100) for 2 consecutive days (b) and mice that received pasteurized kefir (diluted 1/10) for 5 consecutive days (c).

process associated with immune stimulation (Zhou et al. 2000). Takagashi et al. (1991) indicated that probiotic lactobacilli can be readily internalized within M cells of

the gut. However, a recent study (Galdeano & Perdigón, 2004) showed that no entire bacteria are taken up by enterocytes but rather their fragments (bacterial antigenic particles). These studies indicate that translocation of viable bacteria is not required for immunomodulation. There are different ways of internalization of antigenic particles, depending on the uptaking cell involved (M cell *v.* enterocyte). Animal models are of limited value in microbiological risk assessment. The high variability in species-specific responses does not allow the extrapolation of the results to humans. However, immunological effects of probiotics can be assessed in animals and these models are very important in determining dose-response effects (Salminen et al. 1998). In this work, the translocation assay was used as an additional parameter (together with the enumeration of IgA+ and IgG+ cells and haematoxylin-eosin studies of the gut) to determine a dose able to modulate the intestinal immune response without interferences in this animal model that could come from an inflammatory response due to a translocation event.

Numerous reports show the ability of fermented milks (Perdigón et al. 1994; Fukushima et al. 1998; Matar et al. 2001; Cano et al. 2002) or cheese (Medici et al. 2004) to enhance humoral immune response by increasing intestinal IgA. Kefir is reported to enhance the specific intestinal mucosal immune response against cholera holotoxin in young adult but not senescent rats (Thoreaux & Schmucker, 2001). We observed the influence of the dose and cell viability on the capacity of kefir to increase the number of lamina propria IgA+ cells. Although administered 10-times less concentrated, kefir containing viable bacteria achieved a similar effect to that of pasteurized kefir on the number of IgA+ cells. Physical treatment, such as heat-inactivation, may have altered adhesive abilities and affected the immunomodulating capacities of the bacteria in the pasteurized kefir (Ouwehand et al. 2000).

The main function of secretory IgA in the gut is to exert immune exclusion by intimate cooperation with the innate non-specific defence mechanisms. IgG should not be considered an important immunoglobulin at the intestinal mucosa because its external translocation depends on passive intercellular diffusion and increases during intestinal inflammatory processes (Mielants & Veys, 1996; Rodrigues et al. 1998). IgG can activate complement and it may cause an increase of the mucosal permeability and tissue damage (Brantzaeg et al. 1985). In the present study, the functional dose for kefir and pasteurized kefir was chosen by considering the absence of translocation, the capacity of increasing the number of IgA+ cells in the absence of intestinal inflammation (determined by quantifying the lamina propria IgG+ cells) together with the haematoxylin-eosin studies of the small intestine architecture. According to the number of IgA+ and IgG+ cells, 2 d of feeding with kefir diluted 1/100 and 5 d of feeding with pasteurized kefir diluted 1/10 were the doses best able to modulate the intestinal mucosal immune response. Gill (1998), Donnet-Hughes et al. (1999) and Perdigón et al. (1991, 2001) stated

**Table 3.** Effect of the oral administration of kefir (K, diluted 1/100) and pasteurized kefir (PK, diluted 1/10) on the number of IL-2+, IFN $\gamma$ +, TNF $\alpha$ + and IL-12+ cells/10 fields on histological slices of small intestine of mice

Values are the mean  $\pm$  SD of 5 animals/treatment/d

Days of feeding	IL-2		IFN $\gamma$		TNF $\alpha$		IL-12	
	K 1/100	PK 1/10	K 1/100	PK 1/10	K 1/100	PK 1/10	K 1/100	PK 1/10
Control	35.3 $\pm$ 2.1 <sup>a</sup>		37.4 $\pm$ 2.9 <sup>c</sup>		37.3 $\pm$ 2.1 <sup>e</sup>		62.9 $\pm$ 4.9 <sup>g</sup>	
2 d	44.2 $\pm$ 3.7 <sup>b</sup>	50.9 $\pm$ 2.7 <sup>b</sup>	44.3 $\pm$ 5.5 <sup>c</sup>	51.6 $\pm$ 1.5 <sup>d</sup>	42.6 $\pm$ 3.2 <sup>e</sup>	46.8 $\pm$ 3.8 <sup>f</sup>	63.7 $\pm$ 3.3 <sup>g</sup>	80.7 $\pm$ 6.6 <sup>h</sup>
5 d	48.6 $\pm$ 2.2 <sup>b</sup>	49.9 $\pm$ 2.9 <sup>b</sup>	49.7 $\pm$ 4.4 <sup>d</sup>	49.2 $\pm$ 1.5 <sup>d</sup>	43.2 $\pm$ 0.7 <sup>e</sup>	54.4 $\pm$ 2.2 <sup>f</sup>	65.2 $\pm$ 5.2 <sup>g</sup>	86.4 $\pm$ 4.9 <sup>h</sup>
7 d	56.1 $\pm$ 2.9 <sup>b</sup>	52.8 $\pm$ 1.8 <sup>b</sup>	53.2 $\pm$ 2.4 <sup>d</sup>	48.5 $\pm$ 3.9 <sup>d</sup>	52.2 $\pm$ 1.8 <sup>f</sup>	64.7 $\pm$ 5.0 <sup>f</sup>	68.4 $\pm$ 3.0 <sup>g</sup>	88.3 $\pm$ 1.1 <sup>h</sup>

a,b,c,d,e,f,g,h Values in the same column without a common superscript are significantly different from the corresponding control value ( $P < 0.05$ )

that defining the effective dose for any strain is an important step in future studies with probiotics. Our results on the importance of determining a functional dose for kefir agree with these previous findings. Gill & Rutherford (2001) report that both live and heat-killed preparations of *Lactobacillus rhamnosus* HN001 enhance the phagocytic activity of blood and peritoneal leucocytes in mice, but only live lactobacilli enhance gut mucosal antibody responses. These authors state that while the innate cellular immune system is responsive to killed forms of food-borne bacteria, specific gut mucosal immunity may only be stimulated by live forms. Hence, the present results demonstrated the role of orally administered live v. killed bacteria on the balance and the polarization of the intestinal mucosa immune response towards Th1/Th2. This observation was further confirmed by the steady increment of the specific immunoregulatory cytokine IL-10, observed for pasteurized kefir.

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- $\beta$  (Blum et al. 1999). IL-6 promotes terminal differentiation of B cells into plasma cells (Christensen et al. 2002). In our study, IL-4, IL-6 and IL-10 were all significantly increased in the lamina propria of the small intestine of animals that received both types of kefir at the selected dilution, which accords with the increase in the number of IgA+ cells observed and its regulation by viable kefir. IL-4+ and IL-10+ cells steadily decreased throughout the feeding period with kefir, which accords with the decrease in the number of IgA+ cells. On the contrary, IL-10+ cells increased throughout the feeding periods with pasteurized kefir. Since IL-10 is a multifunctional cytokine, we hypothesize that, for pasteurized kefir, the steady increase observed was necessary to control the increment of pro-inflammatory cytokines such as IFN $\gamma$  and TNF $\alpha$ . In the present study, we observed that the Th1 population seemed not to be activated, as confirmed by the Th1 cytokine pattern. However, for the functional dose chosen for pasteurized kefir (5 d of feeding, dilution 1/10), a significant increase of the pro-inflammatory cytokines IFN $\gamma$  and TNF $\alpha$  was observed, but the magnitude of this increment was lower than the one observed for the Th2 immunoregulatory

cytokines (IL-4, IL-6 and IL-10). These results *in vivo* confirm previous findings *in vitro* (Christensen et al. 2002) that show that there are different thresholds of bacterial concentration necessary to induce cytokine production. At the same time, our findings agree with those of Haller & Hammes (1999), who determined that the amount of bacteria necessary to induce maximal TNF $\alpha$  secretion is higher for heat-killed bacteria than for live bacterial cells. IL-12 is an important cytokine for inducing cellular immunity. It enhances the production of Th1 cytokines and the proliferation of Th1 cells (Kato et al. 1999). In contrast, IL-10 is an anti-inflammatory cytokine that suppresses IL-12 production (Christensen et al. 2002). In this study, no IL-12 production was observed when kefir (diluted 1/100) was administered for 2, 5 or 7 consecutive days, probably as an indirect effect of the immunoregulatory characteristics of IL-10. Our experimental results suggest that a Th1 response was controlled by Th2 cytokines induced by kefir feeding. Pasteurized kefir would induce both Th2 and Th1 responses. Our results are also in accord with the statement that, upon oral administration, viable microorganisms are more effective in modulating the gut mucosal immune system, since although kefir was administered 10-times more dilute than pasteurized kefir, it induced an immunomodulation of similar magnitude. There may be application areas where non-viable fermented milks have some health benefits, but little work has been done on non-viable fermented milks. When both fermented products and fermented-then-pasteurized products are able to cause a similar effect, it may not be the bacteria but fermentation products which are responsible for the observed effect (Ouweland & Salminen, 1998). Mainville et al. (2001) showed that different methods for deactivating the bacteria and yeast in kefir introduce changes in the structure of the kefir proteins and lipids as well as in sensory properties and fluid nature of the product. Cell wall integrity is pointed out as one critical factor in determining the immunomodulating capacity of lactic acid bacteria (Vinderola et al. 2004). The host responds to bacterial stimuli by releasing pro-inflammatory or anti-inflammatory cytokines, depending on the strain of bacterium (Cross, 2002). In this work, we also observed that the dose and cell viability played an important role in

the profile of cytokines induced. The targeted effect (a Th1 v. Th2 response) could be achieved by the administration of one or other type of kefir. In this study, we give the first insight into the mechanisms involved in the immunomodulating capacity of the oral administration of kefir containing viable or heat-inactivated bacteria at different doses.

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