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Oral Administration of Kefiran Induces Changes in the Balance of Immune Cells in a Murine Model

Micaela Medrano,[†] Silvia M. Racedo,[†,‡,§] Ivanna S. Rolny,[†,‡] Analía G. Abraham,[†,∥] and Pablo F. Pérez*[,†,‡]

[†]Centro de Investigación y Desarrollo en Criotecnología de Alimentos, CCT La Plata, CONICET, UNLP, 47 y 116 (s/n), La Plata (1900), Argentina

[‡]Cátedra de Microbiología, Facultad de Ciencias Exactas, UNLP, 47 y 115, La Plata (1900), Argentina

[∥]Área Bioquímica y Control de Alimentos, Facultad de Ciencias Exactas, UNLP, 47 y 115, La Plata (1900), Argentina

ABSTRACT: The aim of the present study was to evaluate the effect of the oral administration of kefiran on the balance of immune cells in a murine model. Six week old BALB/c mice were treated with kefiran (300 mg/L) for 0, 2 and 7 days. Kefiran treatment increased the number of IgA+ cells in *lamina propria* after 2 and 7 days. Percentage of B220+/MHCII^{high} cells in mesenteric lymph nodes (2 days) and Peyer's patches (7 days) was higher compared to untreated control mice. An increase of macrophages (F4/80+ cells) was observed in *lamina propria* and peritoneal cavity (2 and 7 days). In contrast, at day 7, macrophage population decreased in Peyer's patches. These results show the ability of kefiran to modify the balance of immune cells in intestinal mucosa. This property could be highly relevant for the comprehension of the probiotic effect attributed to kefir.

KEYWORDS: kefiran, GALT, IgA+ cells, macrophages, intestinal mucosa immune response

INTRODUCTION

Recently, exopolysaccharides (EPS) produced by lactic acid bacteria have gained attention due to their immunomodulating properties.[1,2] This effect could be exerted by stimulation of some cell populations and/or by the induction of cytokine release.

An example of an acid phosphopolysaccharide able to induce mitogenic activity on splenocytes and cells isolated from Peyer's patches is the EPS OLL 1073R-1 produced by *Lactobacillus delbrueckii* subsp. *bulgaricus*.[3] Also, the acid phosphopolysaccharide LA158 isolated from *Lb. gasseri* showed mitogenic activity on B cells.[4] In addition, the phosphopolysaccharide KVS20 from *Lb. lactis* subsp. *cremoris* is able to induce IFN-γ and IL-1 synthesis in murine macrophages[5] and stimulation of TNF-α, IL-6 and IL-12 production was observed with the EPS RW-9595 M produced by *Lb. rhamnosus*.[1] Immunomodulating properties of bacterial EPS are related to their physicochemical characterististics such as monosaccharide composition, number and type of ramifications, molecular weight and tridimensional structure.[2] The role of physicochemical properties on immunomodulating properties of EPS has been demonstrated, and not all EPS are able to stimulate the immune system.[6] Zwitterionic EPS induce memory immune responses and can be presented in the context of MHCII or molecules such as C3.[7,8]

Even though it has been demonstrated that some EPS orally or parenterally administered are able to modulate the immune response, the mechanism involved remains unknown.[2]

Kefiran is the exopolysaccharide produced by microorganisms present in kefir grains or isolated from them.[9] This EPS can be purified from kefir grains, fermented products (milk or whey) or culture supernatants of *Lactobacillus kefiranofaciens*.[10] It is a branched hydrosoluble glucogalactan composed of equal amounts of glucose and galactose with molecular weight higher than 10^7 Da.[11] This EPS also showed interesting physicochemical and functional properties.[12]

Many health promoting effects are attributed to kefiran, i.e. anti-inflammatory, antitumoral, immunomodulatory, hypocholesterolemic and hypotensive activities.[13] Both kefir and kefiran have demonstrated the ability to modify cytokine and immunoglobulin profiles in mice. Vinderola et al.[14] found that kefiran produced by *Lb. kefiranofaciens* (ATCC 43761) orally administered to BALB/c mice leads to an increase in the number of IgA+ cells in small and large intestine *lamina propria* without modifying the number of IgG+ cells. These authors also observed an increase of IL-10+, IL-6+ and IL-12+ cells compared to control mice, whereas the number of IFNγ+ and TNFα+ cells remained unchanged. Serum cytokines also were increased in mice treated with the polysaccharide, and the authors concluded that kefiran is able to induce an immune response thus contributing to intestinal homeostasis.

The aim of the present study was to gain further insight on the effect of oral administration of kefiran on the balance and function of immune cells of the intestinal mucosa and associated lymphoid tissues in a murine model.

MATERIALS AND METHODS

Animals. BALB/c mice (6−8 weeks old) were purchased from Facultad de Ciencias Veterinarias, UNLP (La Plata, Argentina). Each experimental group consisted of 5−7 mice housed in cages that were maintained at controlled temperature and light cycle. Animals were handled in accordance with international regulations for animal welfare.

Isolation and Purification of Kefiran. The water-soluble exopolysaccharide was extracted as previously reported by Rimada et al.[15] Briefly, kefir grains CIDCA AGK1 were treated in boiling

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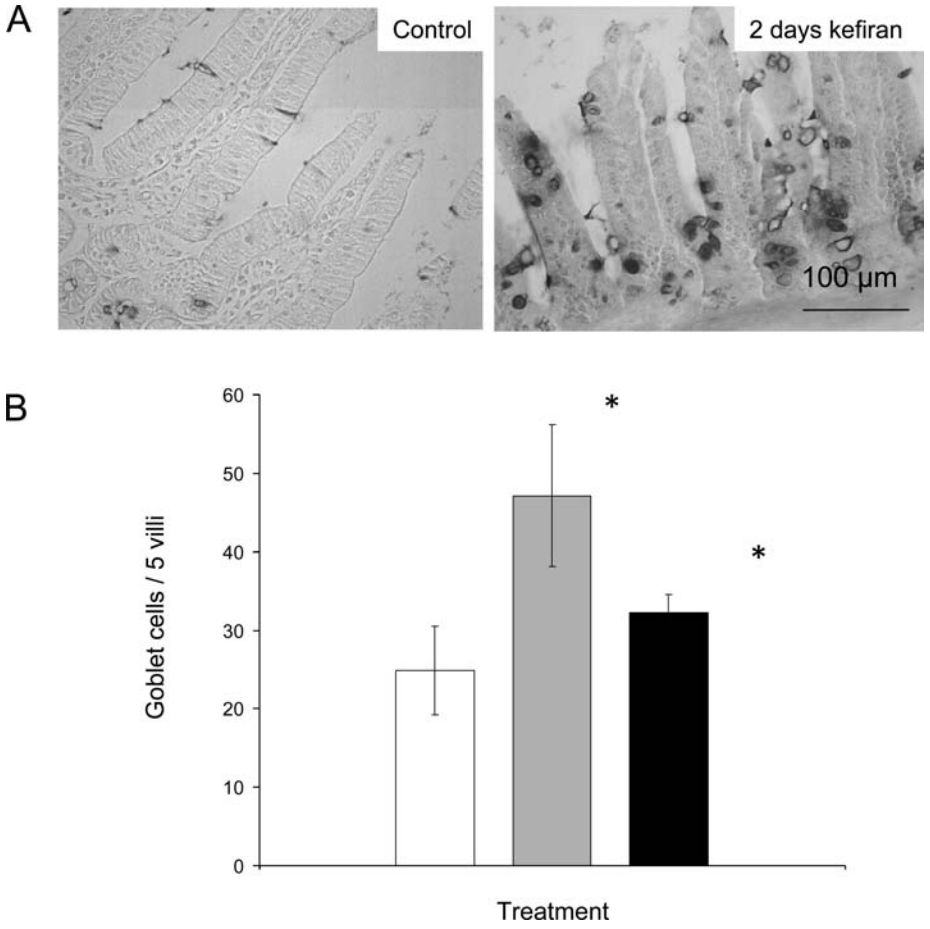

Figure 1. A: Goblet cells in small intestine stained with Alcian Blue of mice control group and treated with kefiran for 2 days. Bar =100 μm. B: Number of goblet cells/5 villi on small intestine epithelium of untreated control mice (white bar) and kefiran treated mice for 2 days (gray bar) and 7 days (black bar). * indicates significant differences (*t* test, $P \leq 0.05$) on the number of goblet cells as compared to control. In panel B, values are averages of 5 determinations and error bars represent standard deviation.

water (1:10 w/v) for 3 h with discontinuous stirring and centrifuged (Sorvall RC-5B Plus centrifuge) at 10000*g* for 20 min at 20 °C. The EPS in the supernatant was precipitated by the addition of two volumes of cold ethanol and stored at −20 °C overnight. The mixture was centrifuged at 10000*g* for 20 min at 4 °C, and pellets were dissolved in hot water. The precipitation procedure was repeated twice. The precipitate was finally dissolved in hot distilled water and lyophilized (Heto FD4, Heto-Holten, Denmark). EPS concentration was determined by the anthrone method, measuring absorbance at 620 nm in a spectrophotometer (Metrolab 330 spectrophotometer, Metrolab, Argentina). Glucose solutions were used as standards.[16] Samples were tested for the absence of free sugars by qualitative thin layer chromatography (TLC) on silica gel G type 60 plates (Merck, D-64271 Darmstadt, Germany) using *n*-propanol−acetic acid−water (70:20:10 v/v/v) as the mobile phase. TLC plates were developed with *p*-aminobenzoic acid 7 g/L and *o*-phosphoric acid 30 g/L in methanol.[17] Protein absence was tested by the Bradford method.[18] Anthrone, Bradford and TLC reagents were obtained from Sigma (St. Louis, MO, USA). Kefiran obtained by this method had purity higher than 99%. Kefiran solutions were prepared by dissolving 300 mg of lyophilized kefiran in 1 L of drinking water and kept at 4 °C until administration to mice. Fresh kefiran solutions were prepared every day.

Feeding Procedures. Mice were fed with a balanced conventional diet. Kefiran (300 mg/L) was administered *ad libitum* in drinking water for 2 or 7 consecutive days to different groups of mice. According to daily water intake (3−4 mL), 0.9 to 1.2 mg of kefiran per mouse per day was administered. Control mice consumed a conventional diet and water *ad libitum*.

Table 1. Percentages of T (CD4+ and CD8+) Cells in Spleen, Mesenteric Lymph Nodes (MLN) and Peyer's Patches of Mice Treated with Kefiran and Control Untreated Animals[a]

sample	treatment	CD8 + cells	CD4+ cells
spleen	control	20.1 ± 6.0	24.2 ± 7.5
	2 days	24.4 ± 10.6	33.4 ± 4.8
	7 days	16.9 ± 3.0	28.4 ± 3.0
MLN	control	21.4 ± 4.6	50.7 ± 8.0
	2 days	25.0 ± 4.5	49.1 ± 3.9
	7 days	17.9 ± 2.5	48.3 ± 6.2
Peyer's patches	control	3.4 ± 1.4	26.0 ± 4.6
	2 days	3.3 ± 1.4	22.6 ± 8.1
	7 days	2.3 ± 1.6	21.4 ± 7.9

[a] Values were calculated from the gates of lymphocytes in the FSC vs SSC plots and represent averages of 5−7 determinations ± standard deviation.

Flow Cytometry Analysis. Mice were euthanized by CO_2 inhalation, and spleen, Peyer's patches and mesenteric lymph nodes (MLN)

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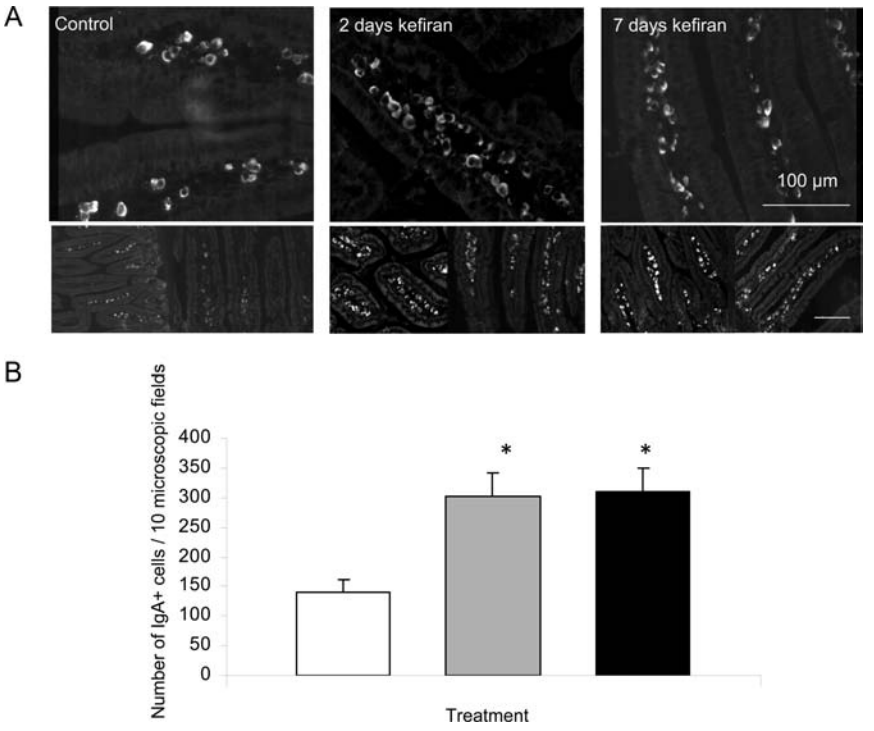

Figure 2. A: IgA+ cells in *lamina propria* of untreated control mice and mice treated with kefiran for 2 and 7 days. Bar =100 μm. B: Number of IgA+ cells in *lamina propria* of control untreated mice (white bar) and treated with kefiran for 2 (gray bar) and 7 days (black bar). * indicates significative differences (*t* test, $P \leq 0.05$) as compared with control untreated animals. In B, values are averages of 5 to 7 determinations and error bars represent standard deviation.

were aseptically removed. Peritoneal macrophages were collected by washing the peritoneal cavity with 2 mL of cold phosphate buffered saline (PBS:KH_2PO_4, 0.144 g/L; Na_2HPO_4, 0.795 g/L; NaCl, 9 g/L; pH, 7). Cell suspensions were obtained by mechanical disruption of tissue, and they were collected in ice-cold phosphate-buffered saline. Samples were centrifuged for 5 min (250*g*) at 4 °C and suspended in PBS supplemented with 2% (v/v) heat-inactivated fetal bovine serum (FBS). Concentration of viable cells was determined by Tripan blue exclusion. Staining was performed as follows: 5×10^5 cells per tube were washed twice with PBS−2% FBS, centrifuged at 250*g* and incubated for 20 min (0 °C) with the appropriate dilution of antibody.

The following monoclonal antibodies were used (eBioscience, San Diego, USA): fluorescein isothiocyanate (FITC) conjugated anti-mouse CD4 (Clone: GK 1.5), phycoerytrin (PE) conjugated anti-mouse CD8 (clone: CT-CD8b), FITC-conjugated anti-mouse/human CD45R (B220) (clone: RA3-6B2), PE conjugated anti-mouse MHC class II (I-A/I-E) (clone: M5/114.15.2), biotin conjugated anti-mouse F4/80 Antigen—Pan Macrophage Marker, BM8-100, CD11c biotin conjugated anti-mouse CD11c (clone: N418), PE conjugated anti-mouse CD86 (B7-2) (clone: PO3.1), PECy5 conjugated anti-mouse CD8α (Ly2) (clone: 536.7). The antibodies were used according to manufacturer's instructions (eBioscience, San Diego, USA). Cells were then washed twice with PBS, fixed in 1% (w/v) paraformaldehyde in PBS and analyzed. Flow cytometry was performed in a FACS-Calibur flow cytometer (Becton and Dickinson, San Jose, CA, USA), and data were analyzed using CellQuest software (BD Biosciences). A total of 10000 events/sample were collected. Appropriate isotype controls were used.

Preparation of Histological Sections of Small Intestine. Mice were euthanized by CO_2 inhalation, and the small intestine was aseptically removed. Samples were fixed in absolute ethanol, dehydrated and embedded in Histowax (Leica Microsystems Nussloch GmbH) according to Sainte-Marie.[19] Samples were stained with hematoxylin−eosin or Alcian Blue−eosin.

IgA+ and F4/80+ cells in *Lamina Propria* and Peyer's Patches. Histological sections were deparaffinized and rehydrated in a graded series of ethanol and water and finally washed with PBS. Nonspecific protein binding was minimized by treatment for 30 min at room temperature with PBS/10% FBS (v/v). The number of IgA producing cells and macrophages F4/80+ was determined by using the respective monospecific antibodies: anti-mouse IgA (α-chain specific) biotin conjugated (Sigma-Aldrich, St. Louis, MO, USA) and anti-mouse F4/80 biotin conjugated (Pan Macrophage Marker, eBioscience, San Diego, CA, USA). The binding of primary antibody was detected by using streptavidin−FITC (Sigma-Aldrich). Histological samples were incubated with the appropriate antibody for 30 min at 37 °C. Afterward, samples were examined by fluorescence microscopy in a LEICA DMLB (Leica Microsystems, Wetzlar GmbH, Germany) coupled to a DC100 camera (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland). The number of positive cells in 10 microscopic fields (63× magnification) was evaluated in 3 specimens per mouse in groups of 5 mice per treatment and time point. Slides were coded and evaluated by an operator unaware of the experimental conditions.

Statistical Analysis. Results were compared by means of the two-tailed Student's *t* test (InfoStat version 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

RESULTS

Histological Study. Histological sections of small intestine stained with hematoxylin−eosin showed no changes on intestinal mucosa structure of mice treated during 2 and 7 days with kefiran compared to control group (data not shown). No evidence of inflammatory processes was observed.

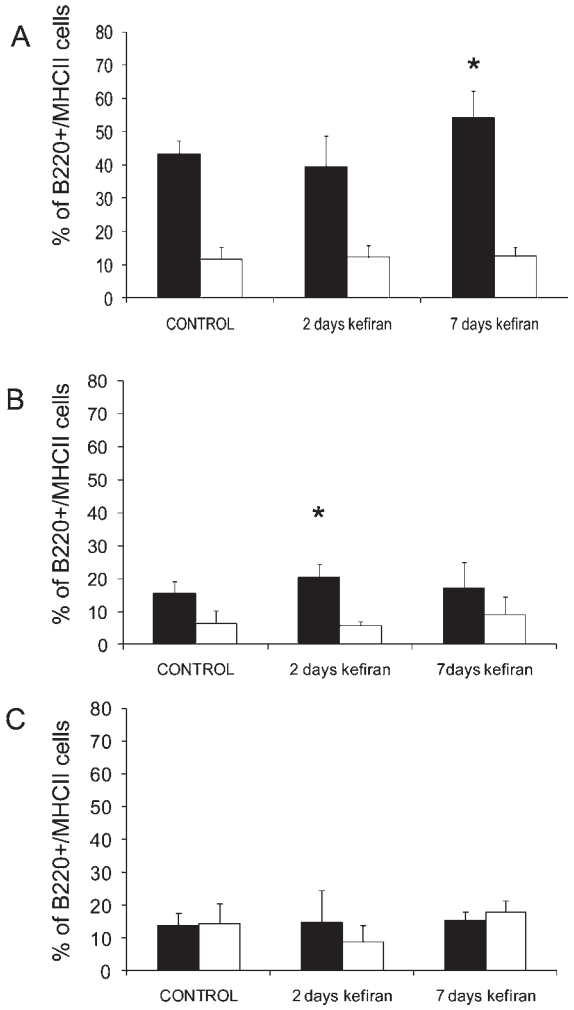

Figure 3. MHCII^{low} (white bar) and MHCII^{high} (black bar) expression in B220+ cells isolated from PP (A), MLN (B) and spleen (C) of control mice and mice treated with kefiran for 2 and 7 days. * indicates significant differences (t test, $P \leq 0.05$) on MHCII^{high} expression between treatments and control group. Values are averages of 5 to 7 determinations, and error bars represent standard deviation.

A significant ($P \leq 0.05$) increment of mucus producing cells (goblet cells) was observed in the small intestine of mice treated with kefiran (Figure 1).

T Cells. Effect of kefiran on T-cell populations (CD4+ and CD8+) was evaluated by flow cytometry in Peyer's patches (PP), mesenteric lymph nodes (MLN) and spleen of mice treated with kefiran for 2 and 7 days or control untreated mice. There were no significant differences in CD4+ and CD8+ cells between treated and untreated mice in the analyzed tissues (Table 1).

B Cells. *IgA+ Cells in Lamina Propria.* Histological sections of the small intestine labeled with anti-IgA antibody were observed by fluorescence microscopy (Figure 2A). Number of IgA+ cells was determined in 10 random microscopical fields. Treatment of mice with kefiran for 2 and 7 days significantly increases the number of IgA+ cells as compared with control untreated animals (Figure 2B).

MHCII Expression in B 220+ Cells. Expression of MHCII is shown in Figure 3. A significant increase ($P \leq 0.05$) on the percentage of B220+/MHCII ^{high} cells in PP was observed in mice treated with kefiran for 7 days as compared to control untreated mice group (Figure 3A). This analysis also showed a significant increment ($P \leq 0.05$) in B220+/MHCII ^{high} cells in MLN of mice treated for 2 days with kefiran as compared to control group (Figure 3B). In spleen, there were no differences in the studied cell populations between control and kefiran fed mice (Figure 3C). It is worth noting that percentage of B cells (B220+) was not modified by kefiran administration. Indeed, average values for this cell population in tissues of treated or control untreated animals, calculated on the basis of lymphocyte gate on SSC vs FSC plots, were 33.4 ± 1.7, 28.3 ± 1.5 and 60.4 ± 3.8 for spleen, MLN and Peyer's patches respectively.

Table 2. Percentage of Total Dendritic Cells (CD11c) and Activated Dendritic Cells (CD11c+/CD86+) in Peyer's Patches and Mesenteric Lymph Nodes (MLN)

sample	treatment	CD11c+[a]	CD11c+/CD86+[a]
Peyer's patches	control	1.9 ± 0.5	7.7 ± 0.6
	2 days	2.2 ± 0.4**	5.0 ± 0.9*
	7 days	2.5 ± 0.5**	4.8 ± 0.5*
MLN	control	0.9 ± 0.4	11.4 ± 2.6
	2 days	0.9 ± 0.4	11.2 ± 2.4
	7 days	1.1 ± 0.2**	8.8 ± 2.3**

[a] Total dendritic cells (CD11+ cells) are referred to total gated cells in the FSC vs SSC plot. Activated dendritic cells (CD11+/CD86+ cells) are referred to total CD11+ cells. Values are averages of 5 to 7 determinations ± standard deviation. * indicates significant differences (t test, $P \leq 0.05$) as compared with control untreated animals. ** indicates trend (t test, $P \leq 0.08$) as compared with control untreated animals (0 days).

Dendritic Cells and Macrophages. *Total Dendritic Cells (CD11c+).* Cells obtained from PP and MLN were labeled with anti-mouse CD11c-FITC to evaluate the number of total dendritic cells (CD11c+). A trend ($P = 0.08$) of higher ratio of CD11c+ cells after 7 days of kefiran administration was observed (Table 2).

Subpopulations of Dendritic Cells. Dendritic cell subpopulations CD8α+ (lymphoid), CD11b+ (myeloid) and CD11b−/CD8α− were analyzed in different tissues by specific antibody labeling and flow cytometry. There were no significant differences between kefiran fed mice and control group. Average percentages, calculated on the basis of total CD11c+ cells, were 29.0 ± 1.4 (CD8α+, PP), 21.4 ± 2.9 (CD11b+, PP), 49.6 ± 3.5 (CD8α−/CD11b−, PP), 64.7 ± 5.4 (CD8α+, MLN), 40.2 ± 4.8 (CD11b+, MLN) and 0.0 (CD8α−/CD11b−, MLN).

Activated Dendritic Cells (CD11c+/CD86+). To evaluate the percentage of activated dendritic cells in PP and MLN, cells were double labeled with anti-CD11c-FITC and anti-CD86-PE and analyzed by flow cytometry.

A significant diminution on CD11c+/CD86+ cells was observed in PP after 2 and 7 days of kefiran treatment compared to control group (Table 2). No significant differences were observed in MLN or spleen. There were trends ($P = 0.08$) to a decrease in the ratio of activated dendritic cells in MLN after 7 days of kefiran treatment (Table 2).

Macrophages (F4/80+). To study F4/80+ cells in *lamina propria* of the small intestine, histological sections of kefiran-fed and control mice were labeled with anti-F4/80 and observed by fluorescence microscopy. As shown in Figure 4, kefiran treatment for 2 and 7 days leads to a significant increment in the number of F4/80+ cells in *lamina propria* as compared to control untreated animals. Values of F4/80+ cells in 10 random microscopic fields are shown in Table 3 A.

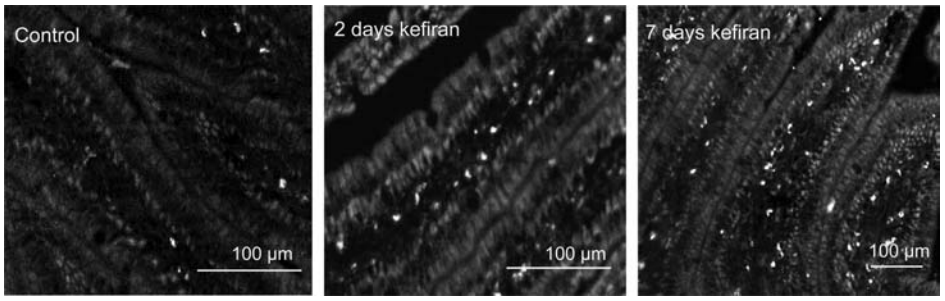

Figure 4. Representative immunofluorescence micrographs showing F4/80+ cells in *lamina propria* from control mice and treated with kefiran for 2 and 7 days. Bar =100 μm.

Table 3. Macrophages (F4/80+) in *Lamina Propria*, Peritoneal Cavity and Peyer's Patches[a]

	control	2 days	7 days
	A: F4/80+ Cells[b]		
lamina propria	55.7 ± 8.3	149.7 ± 28.0*	140.0 ± 20.1*
	B: F4/80+ cells[c]		
Peyer's patches	22.0 ± 15.7	21.8 ± 15.8	8.4 ± 4.1*
peritoneal cavity	43.9 ± 10.9	42.6 ± 7.8	61.6 ± 9.3*

[a] Values are averages of 5 to 7 determinations ± standard deviation. * indicates significant differences (t test, $P \leq 0.05$) between treatments and control group. [b] In panel A values represent the number of F4/80+ cells in 10 random microscopical fields (63×). [c] In panel B, values represent the percentages of F4/80+ cells from the total cell population gated in a FSC vs SSC plot (flow cytometry).

Macrophage population was also studied in PP by flow cytometry. A significant decrease ($P < 0.05$) was observed after 7 days of kefiran treatment (Table 3B).

Concerning peritoneal macrophages, a significant increase ($P \leq 0.05$) of F4/80+ cells was observed in peritoneal cavity after 7 days of kefiran treatment (Table 3B).

DISCUSSION

The present work shows that oral administration of kefiran isolated from kefir grains is able to modify the balance of cell populations in different regions associated with the mucosal immune system as well as at a systemic level.

Our results demonstrate that kefiran induces early changes after oral administration. It is worth noting that, even at day 2, there were modifications in cell populations of LP (increase of F4/80 and IgA+ cells), PP (increase of total CD11c+ cells and decrease of CD11c+/CD86+ cells) and MLN (increase in the ratio of B220+/MHCII^{high} cells). Effects found at day 2 in MLN suggest that kefiran starts to modify the ratio of cell populations even before the first time point of the study (2 days). The raise in IgA+ cells in LP at day 2 indicates that class switching has already occurred. It is known that class switching depends on the presence of factors such as vasoactive intestinal peptide, BLys, APRIL, TGF-β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-15 and IFN-γ.[20] These factors can be autocrine and/or secreted by different cell types, e.g. T-cells and dendritic cells. These results are in agreement with those reported by Vinderola et al.[14] with kefiran isolated from *Lactobacillus kefiranofaciens*. It is also possible that the increase of macrophages in *lamina propria* facilitates antigen presentation for T-dependent antigens thus leading to an increase in IgA+ cells.

Dendritic cells (DC) are key players at the interface between innate and adaptive immunity. It has been demonstrated that CD11c+ cells in Peyer's patches that express costimulatory molecules such as CD86 and MHCII are efficient for antigen processing and presentation.[21,22] In the present study, we demonstrate that in Peyer's patches of animals treated with kefiran, there was an increase of dendritic cells (DC) at days 2 and 7. Interestingly, activated DC (CD11c+/CD86+) decreased in this tissue at days 2 and 7 of kefiran administration. These findings are compatible with a recruitment of DC in PP with no further activation.

In *lamina propria*, there is an increase of macrophages (F4/80+ cells) both at 2 and at 7 days of kefiran administration. This result is also compatible with cell recruitment. Of note, no evidence of either structural modifications or diminution of the villus/crypt ratio was observed, thus indicating lack of an inflammatory response. Interestingly, we found an increase of IgA+ cells in *lamina propria* that correlates with the increase of B cells with high expression of MHCII in PP. The increase of B cells B220+/MHCII ^{high} in PP after kefiran administration is related to the role of Peyer's patches both as inductive and as effector sites of the immune response.[23]

Noteworthy, we found an increase of goblet cells in the villus of the kefiran-treated mice. This finding probably indicates a stimulation of cell proliferation and differentiation of crypt cells that could lead to a mucus-rich environment that, along with the increase in IgA+ cells, gives a layer with high concentration of IgA thus contributing to the defense of the epithelium.[24]

After interaction of kefiran with intestinal mucosa, effects are found in mesenteric lymph nodes where there was an increase in DC and a decrease in activated DC (CD86+) at day 7. We can hypothesize that there is a mobilization of DC from PP to MLN.

The increase of peritoneal macrophages reveals that induction of the immune response by kefiran in the intestinal context leads to a systemic effect that modulates response in different localizations. Therefore, results published by Vinderola et al.[25] showing the increment on the *ex vivo* phagocytic activity of peritoneal and lung macrophages in mice treated with kefir could be ascribed, at least in part, to the presence of kefiran.

Our results are in agreement with the role of polysaccharides as T-independent antigens. Indeed, we found no changes in T-cell populations but there was an increase of B220+/MHCII^{high} cells in Peyer's patches.

The decrease of macrophages (F4/80+) in Peyer's patches at day 7, concomitant with the increase of CD11+ cells and the

decrease of CD86+ cells, is compatible with two scenarios: mobilization of macrophages form Peyer's patches to *lamina propria* or a shift in the differentiation of monocytes to dendritic cells instead of differentiation to macrophages.

Results of the present study were obtained in mice that were only treated with kefiran. Even though no extrapolation can be done for systems challenged with an additional stimulus (e.g., infection), our results encourage further research on the role of kefiran in the prophylaxis and treatment of intestinal pathologies.

AUTHOR INFORMATION

Corresponding Author

*Phone/fax: 54 221 424 9287 E-mail: pfp@biol.unlp.edu.ar.

Present Addresses

[§]Unité Propre de Recherche Microbiologie et Biochimie Appliquée de la Ecole Nationale de Ingenieurs de Travaux Agricoles de Bordeaux, Université de Bordeaux, France.

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