Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity

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Abstract

The probiotic effects ascribed to lactic acid bacteria (LAB) and their fermented dairy products arise not only from whole microorganisms and cell wall components but also from peptides and extracellular polysaccharides (exopolysaccharides) produced during the fermentation of milk. There is a lack of knowledge concerning the immune mechanisms induced by exopolysaccharides produced by lactic acid bacteria, which would allow a better understanding of the functional effects described to them. The aim of this study was to investigate the in vivo immunomodulating capacity of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* by analyzing the profile of cytokines and immunoglobulins induced at the intestinal mucosa level, in the intestinal fluid and blood serum. BALB/c mice received the exopolysaccharide produced by *L. kefiranofaciens* for 2, 5 or 7 consecutive days. At the end of each period of administration, control and treated mice were sacrificed and the numbers of IgA+ and IgG+ cells were determined on histological slices of the small and large intestine by immunofluorescence. Cytokines (IL-4, IL-6, IL-10, IL-12, IFNγ and TNFα) were also determined in the gut lamina propria as well as in the intestinal fluid and blood serum. There was an increase of IgA+ cells in the small and large intestine lamina propria, without change in the number of IgG+ cells in the small intestine. This study reports the effects of the oral administration of the exopolysaccharide produced by *L. kefiranofaciens* in the number of IgA+ cells in the small and large intestine, comparing simultaneously the production of cytokines by cells of the lamina propria and in the intestinal fluid and blood serum. The increase in the number of IgA+ cells was not simultaneously accompanied by an enhance of the number of IL-4+ cells in the small intestine. This finding would be in accordance with the fact that, in general, polysaccharide antigens elicit a T-independent immune response. For IL-10+, IL-6+ and IL-12+ cells, the values found were slightly increased compared to control values, while IFNγ+ and TNFα+ cells did not change compared to control values. The effects observed on immunoglobulins and in all the cytokines assayed in the large intestine after kefiran administration were of greater magnitude than the ones observed in the small intestine lamina propria, which may be due to the saccharolytic action of the colonic microflora. In the intestinal fluid, only IL-4 and IL-12 increased compared to control values. In blood serum, all the cytokines assayed followed a pattern of production quite similar to the one found for them in the small intestine lamina propria. We observed that the exopolysaccharide induced a gut mucosal response and it was able to up and down regulate it for protective immunity, maintaining intestinal homeostasis, enhancing the IgA production at both the small and large intestine level and influencing the systemic immunity through the cytokines released to the circulating blood.
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1. Introduction

Fermented milks and their related dairy lactic acid bacteria (LAB) have demonstrated health benefits [1–5] and therefore are functional products. Probiotics are defined as 'live microorganisms which when consumed in adequate numbers confer a health benefit on the host beyond basic nutrition'. Microorganisms may impart health-promoting characteristics in food through the production of bioactive metabolites (referred to as biogenics) during fermentation [6]. The probiotic effects ascribed to LAB and fermented dairy products arise not only from whole microorganisms and cell wall components, but also from metabolites such as peptides and extracellular polysaccharides produced during the fermentation. The structures of these exopolysaccharides have been studied in detail, but there have been few studies on the physiological functions of the exopolysaccharides [7]. Kefir is a fermented milk drink produced by the action of LAB, yeasts and acetic acid bacteria, trapped in a complex matrix (kefir grains) of microorganisms, exopolysaccharides and proteins [8]. Early observations of the structure of kefir grains noted that some of the bacteria were encapsulated by an exopolysaccharide called kefiran [9]. Several bacteria isolated from kefir are able to produce the heteropolysaccharide kefiran, including *Lactobacillus kefiranofaciens*, *L. kefirgranum*, *L parakefir*, *L. kefir* and *L. delbrueckii* subsp. *bulgaricus* [8,10,11].

The immunomodulating capacity of exopolysaccharides produced by LAB such as *Lactococcus lactis* subsp. *cremoris*, *L. delbrueckii* subsp. *bulgaricus* and *Leuconostoc mesenteroides* has been previously studied. It was shown that some exopolysaccharides derived from these LAB possess B-cell mitogen activity [12–14], the capacity to induce cytokine production [15] and the capacity to modify some macrophage and splenocyte functions [16,17]. In particular for kefiran, there are reports about its antimicrobial and wound-healing properties [18,19], its capacity to lower blood pressure and cholesterol in serum [20,21] and its capacity of retarding tumor growth in some experimental models [22–25]. However, there still exists a lack of knowledge concerning the immune modulation induced by exopolysaccharides produced by LAB, which would allow a better understanding of the functional effects described for them.

We recently demonstrated the immunomodulating capacity of kefir in a murine model, showing the importance of the dose and cell viability to obtain a Th2 or Th1 response [26]. Kefir has the capacity of increasing the phagocytic activity of peritoneal and pulmonary macrophages and to modulate the mucosal response at distant sites [27]. We also observed the effects of kefir microflora and the non-bacterial fraction on cytokine production by cells of the innate immunity-adherent populations of Peyer's patches and the peritoneal macrophages [28]. Since kefir drink contains kefiran, it was of interest to determine the effect of this exopolysaccharide on immune function. The aim of this work was to study the in vivo immunomodulating capacity of the exopolysaccharide produced by *L. kefiranofaciens* by analyzing the profile of cytokines and immunoglobulins induced at the intestinal mucosa level in mice.

2. Materials and methods

2.1. Bacteria and fermentation conditions

Lactobacillus kefiranofaciens (ATCC 43761, American Type Culture Collection, Manassas, VA, USA) was grown in Lactic Acid Whey broth (LAW, ATCC) prepared as follows. 100.0 g of skim milk powder (Agropur, Granby, QC, Canada) was suspended in 500 ml distilled water. The reconstituted milk was adjusted to pH 5.5 with liquid DL-lactic acid syrup (85%, Sigma Chemical Co., St. Louis, MO, USA). The volume was brought to 1 l with additional distilled water and boiled for 30 min. The precipitate was pelleted by centrifugation (15000g; 25 min; 4 °C) and the supernatant volume was returned to 1 l with distilled water. The LAW broth was used to resuspend the following (g/l) : yeast extract, 5.0; trypticase peptone (Difco), 10.0; tryptose (Difco), 3.0; sodium acetate, 2.0; ammonium citrate, 2.0; KH_2PO_4, 0.6; $MgSO_4$, 0.5; $MnSO_4$, 0.5; and Tween 80, 1.0 ml. The pH was adjusted to 5.5 with liquid DL-lactic acid (Sigma) or 1 N KOH before autoclaving at 121 °C for 15 min.

Before *L. kefiranofaciens* was used for inoculation, it was propagated and transferred twice into fresh LAW-medium. Each of the transfers was carried out after 24 h of growing. Fermentations were carried out at 25 °C in 500 ml bottles under anaerobic (85% nitrogen, 10% hydrogen, 5% carbon dioxide) conditions. The pH was measured daily and adjusted to pH 5.5 with 6 N KOH.

2.2. Extraction and purification of the exopolysaccharide

Broth fermented by *L. kefiranofaciens* was cooled on ice, diluted (broth:water, 1:2) with deionized water, and then heated at 100 °C for 15 min to inactivate enzymes and kill all bacteria. Cells were separated using centrifugation (9500g; 20 min; 4 °C), and the exopolysaccharide was precipitated from the supernatant overnight at 4 °C by adding two volumes of 95% ethanol. The precipitate was concentrated using centrifugation (9500g; 20 min; 4 °C). The kefiran was then dissolved in distilled water (70 °C) and then dialyzed against distilled water (dialysis membrane: cutoff 12000–14000 Da; Spectra/Por® Thomas Scientific, USA) at 4 °C for 48 h, with two changes of water. The concentrated kefiran solution was frozen and then lyophilized (Freezone 4.5, Labconco, Kansas City, USA).

2.3. Animals and feeding procedures

Six- to 8-week-old BALB/c female mice weighing 20–25 g were obtained from Charles River (Montreal, Canada). A completely randomized design was chosen for this study. Each experimental and control group

consisted of five mice housed together in plastic cages kept in a controlled atmosphere (temperature 22 ± 2 °C; humidity $55 \pm 2\%$) with a 12 h light/dark cycle. Mice were maintained and treated in accordance with the guidelines of the Canadian Council on Animal Care.

Kefiran was dissolved in phosphate buffered saline (PBS, pH 7.4) solution (Sigma–Aldrich, St. Louis, MO, USA). Treated mice received by gavage 100 mg/kg body weight/day of kefiran for 2, 5 or 7 consecutive days. Control mice received the same volume of PBS instead. All groups of mice, and a control group, received simultaneously a conventional balanced diet *ad libitum* and water. Test and control animals were sacrificed after 2, 5 or 7 days of exopolysaccharide administration.

2.4. Histological studies of the gut

After each administration period, treated and control animals were anesthetized and sacrificed by cervical dislocation. The small (jejune and ileum) and large (ascendant colon to rectum) intestines were removed for histological preparation following the Sainte-Marie technique [29] for paraffin inclusion. Histological slices (4 μm) were stained with haematoxilin–eosin followed by light microscopy examination (magnification 400×).

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

The number of IgA and IgG producing (IgA+ and IgG+) cells was determined on histological slices from the small and large intestine by a direct immunofluorescence method. The immunofluorescence test was performed using (α-chain specific) anti-mouse IgA FITC conjugate or (γ-chain specific) anti-mouse IgG FITC conjugate (Sigma–Aldrich, St. Louis, MO, USA). 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 100×). Data represent the mean of three histological slices for each animal, for each sampling day (five animals per group).

2.6. Cytokine determination in lamina propria of small and large intestine

IL-4, IL-6, IL-10, IFNγ and TNFα were studied by an indirect immunofluorescence method on histological slices from the small and large intestine lamina propria. Histological slices were deparaffinized and rehydrated in a graded series of ethanol, and then incubated for 30 min in a 1% blocking solution of bovine serum albumin (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-4, IL-6, IL-10, IFNγ or TNFα (Peprotech, Inc., Rocky Hill, NJ, USA) polyclonal antibodies (diluted 1/100 in PBS solution). The incubation was followed by two 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) diluted 1/100 in PBS solution, 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+, IFNγ+ or TNFα+ cells (positive = fluorescent cell) per 10 fields (magnification 100×). Data represent the mean of three histological slices for each animal, for each sampling day (five animals/sampling day).

2.7. Cytokine determination in serum and intestinal fluid

Blood from treated or control animals was obtained from the heart of anesthetized animals, allowed to stand for 1 h at 37 °C, centrifuged (6000*g*, 4 °C 10 min) and the blood serum was kept frozen until use. The small intestine of treated or control animals was flushed with 5 ml of PBS and this fluid was centrifuged (10,000*g*, 4 °C 10 min) to separate particulate material. The supernatant was kept frozen until use. IL-4, IL-6, IL-10, IFNγ and TNFα were determined in blood serum and intestinal fluid using the corresponding mouse ELISA Set (BD OptEIA, BD Biosciences PharMingen, San Diego, CA, USA).

2.8. 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 $p < 0.05$.

3. Results

The mucosal immunomodulating capacity of the kefiran produced by *Lactobacillus kefiranofaciens* ATCC 43761 was assessed in this study by the examination of its effects on the IgA+ and IgG+ B cell populations and cytokines from the gut mucosa and on the cytokines in the circulating blood and small intestine fluid.

The histological study of the small and large intestine of mice that received kefiran for 2, 5 or 7 consecutive days showed no lymphocyte infiltrates, nor the presence of oedema or mucosal atrophy, compared to control mice. No significant morphological changes in the overall architecture of the small or large intestine tissue were observed when compared to control mice (figures not shown). The effects of kefiran administration on the IgA+ and IgG+ B cell populations and cytokine+ cells in the small intestine mucosa are shown in Fig. 1. Fig. 1 shows an histological slice of the small intestine where fluorescent IgA+ B cells can be observed in the lamina propria of control
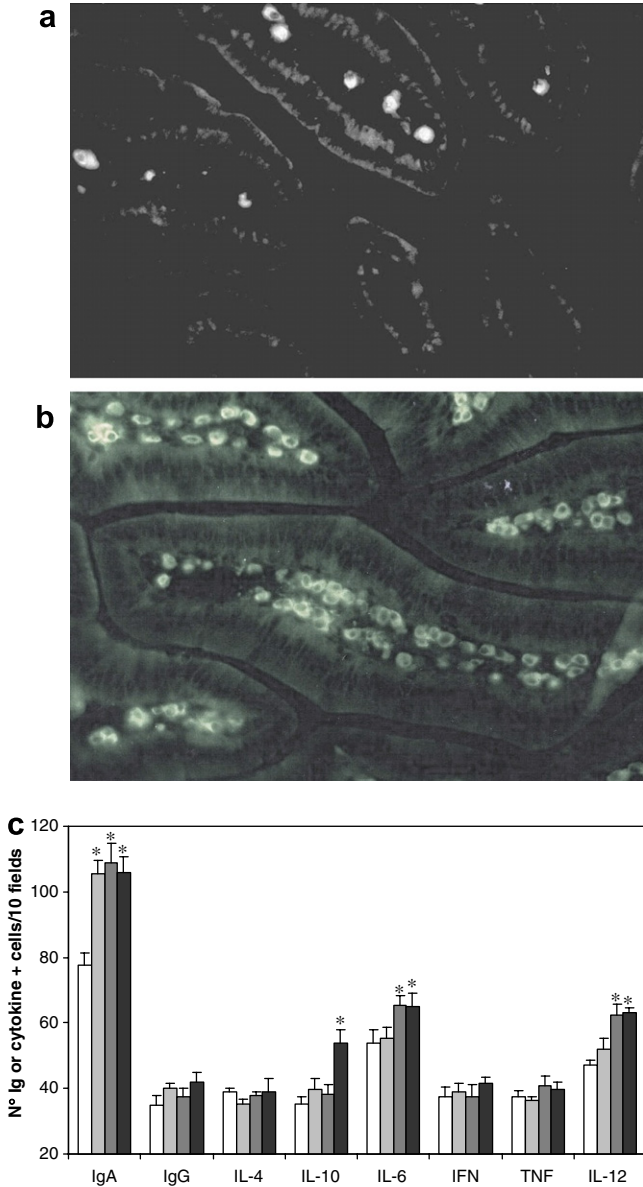
Fig. 1. Effects of kefiran on the small intestine. Histological slice of the small intestine showing fluorescent IgA+ cells in the lamina propria of control (a) or treated (b) mice. (c) Number of IgA+, IgG+, IL-4+, IL-10+, IL-6+, IFNγ+, TNFα+ and IL-12+ cells in the lamina propria of the small intestine of mice that received kefiran for 2 (▨), 5 (▨) or 7 (■) consecutive days, compared to control mice (□). *Statistically different from the corresponding control value ($p < 0.05$).

(Fig. 1a) or in animals that received the exopolysaccharide for 7 days (Fig. 1b). The number of IgA+ cells increased for all periods of administration assessed, while the number of IgG+ cells did not change when compared to control values (Fig. 1c). As regards to the number of cytokine+ cells, the number of IL-6+ and IL-12+ cells increased after 5 or 7 days of kefiran administration while the number of IL-10+ cells increased only after 7 days in relation to control mice. For IL-4+, IFNγ+ and TNFα+ cells, the values obtained from kefiran treated mice were similar to control values (Fig. 1c).
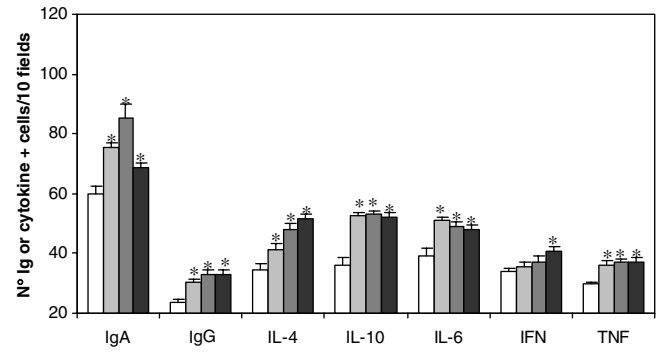

Fig. 2. Effects of kefiran on the large intestine. Number of IgA+, IgG+, IL-4+, IL-10+, IL-6+, IFNγ+ and TNFα+ cells in the lamina propria of the large intestine of mice that received kefiran for 2 (▨), 5 (▨) or 7 (■) consecutive days, compared to control mice (□). *Statistically different from the corresponding control value ($p < 0.05$).

Fig. 2 shows the immune effects of the exopolysaccharide kefiran on the large intestine lamina propria. In general, the effects observed here were more widespread than in the small intestine. All the immune parameters analyzed: the number of IgA+, IgG+, IL-4+, IL-10+, IL-6+ and TNFα+ cells increased for all periods of administration, compared to control values. However, the number of IFNγ+ cells increased (significantly compared to control) only after 7 days of kefiran administration.
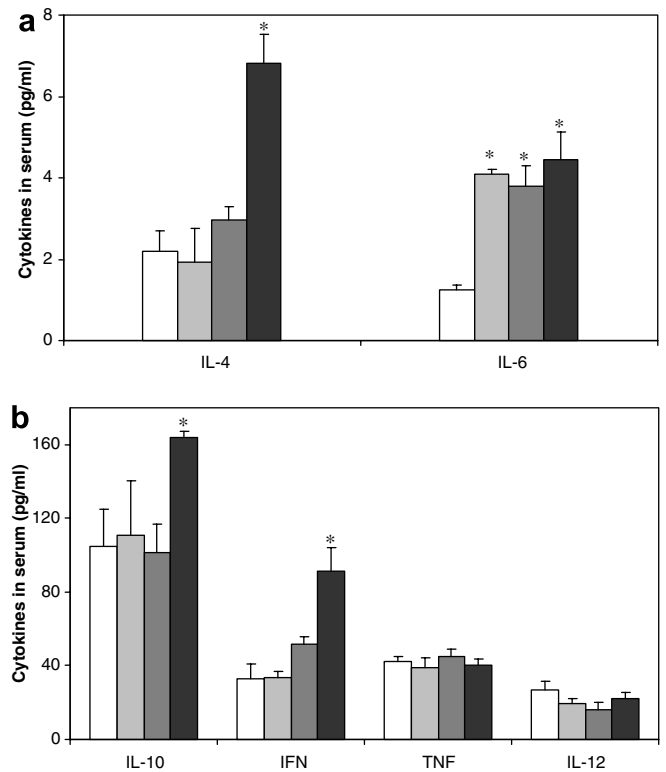

Fig. 3. Effects of kefiran on serum cytokines. IL-4, IL-6 (a) and IL-10, IFNγ and TNFα (b) in the serum of mice that received kefiran for 2 (▨), 5 (▨) or 7 (■) consecutive days, compared to control mice (□). *Statistically different from the corresponding control value ($p < 0.05$).
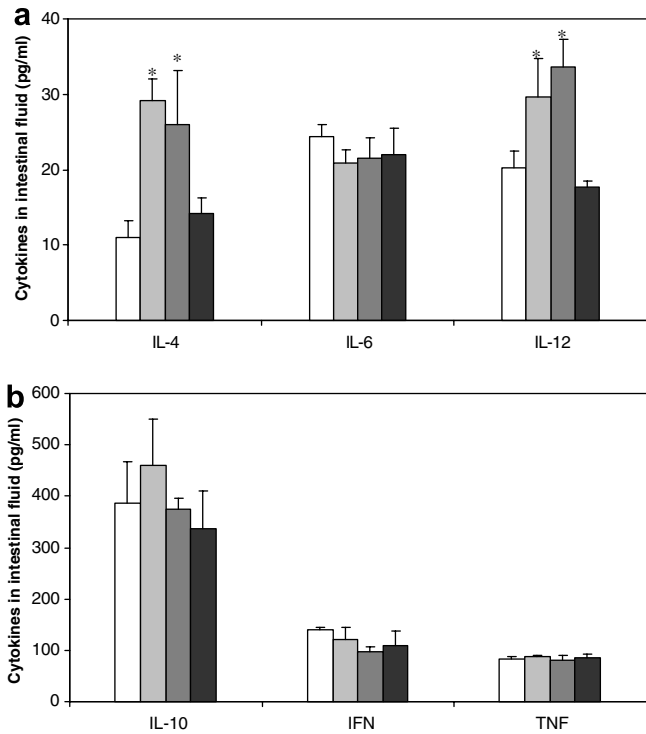
Fig. 4. Effects of kefiran on small intestine fluid cytokines. IL-4, IL-6, IL-12 (a) and IL-10, IFNγ and TNFα in the serum of mice that received kefiran for 2 (■), 5 (■) or 7 (■) consecutive days, compared to control mice (□). *Statistically different from the corresponding control value ($p < 0.05$).

There was an increase of IL-6 for all treatment periods assessed in the blood serum of animals that received kefiran and an increase of IL-4, IL-10 and IFNγ for the 7-day period of kefiran administration. The TNFα and IL-12 content in blood serum did not change when compared to control mice for the same periods of administration. These results are shown in Fig. 3.

The cytokines measured in the intestinal fluid showed only an increase in the content of IL-4 and IL-12 at days 5 and 7 of kefiran administration, compared to control mice, while levels of IL-6, IFNγ and TNFα remained unchanged compared to their respective controls (Fig. 4).

4. Discussion

The exopolysaccharide present in kefir was first studied by La Rivière et al. [9], who named it kefiran. Detailed nuclear magnetic resonance (NMR) analysis showed that the water soluble exopolysaccharide consisted of approximately equal proportions of glucose and galactose. Kefiran is an exopolysaccharide encapsulating some kefir bacteria. Toba et al. [31] isolated an exopolysaccharide producing bacteria from kefir which they named *Lactobacillus kefiranofaciens*. In this paper, we studied the immunomodulating capacity of kefiran, the exopolysaccharide produced by *Lactobacillus kefiranofaciens*, a species commonly found among kefir microbiota [8,20].

Sugars have long been described as T-cell independent antigens that fail to induce immunologic memory and

immunoglobulin class-switching [32]. The prevailing theory is that T-independent antigens bind to polysaccharide-specific B cells and stimulate production of specific antibodies without recruitment of T cell help or induction of immunological memory [33]. In this work, we demonstrated that the oral administration of kefiran to mice, at a dose of 100 mg/kg of body weight, induced an increase in the number of IgA+ cells in the small and large intestine lamina propria, without modification of the number of IgG+ cells in the small intestine. In the gut, IgA+ cells and secretory IgA play an important role as the first line of defense in the host. This immunoglobulin exerts immune exclusion by intimate cooperation with the innate non-specific defense mechanisms [34]. There have been reports of antibody production at the systemic level after the oral administration of exopolysaccharides [35,36]. The role of the non-bacterial fraction of kefir in immune stimulation was also reported [28]. This study is the first to report the effect on the IgA+ cells and cytokines in the small and large intestine following the administration of the exopolysaccharide produced by the strain of *L. kefiranofaciens*. IgM+ B cell immunoglobulin switching and differentiation to IgA+ cells or plasmocytes secreting IgA occurs in an environment rich in IL-4, IL-5 and TGF-β [37], while IL-6 promotes terminal differentiation of B cells into plasma cells [38]. In this study, the increase in the number of IgA+ cells was not simultaneously accompanied by an enhance of the number of IL-4+ cells, however it was accompanied by an enhance of IL-6+ cells in the small intestine, as observed for example in previous works when kefir, containing viable or non-microorganisms or its non-bacterial fraction, was administered [26–28]. These findings would be in accordance with the fact that, in general, polysaccharides antigens elicit a T-independent immune response [39]. However, it was recently reported that polysaccharides might also behave as T-dependent antigens [40]. For T-independent antigens, most of the source of mucosal IgA is from peritoneal B1 cells [41].

In our study, some cytokine+ cells were enhanced in the small intestine after kefiran administration: the number of the pro-inflammatory cytokines IL-6+ and IL-12+ cells increased at days 5 and 7 after kefiran administration. It is known that macrophages and dendritic cells of the lamina propria, among other cell types, are producers of these cytokines [42]. They could be then the source of enhanced IL-6 and IL-12 in the small intestine lamina propria. The increase in the number of the regulatory IL-10+ cells at day 7 might be involved in the control of the intestinal homeostasis after the administration of a dietary antigen. When considering the effects observed in the small intestine, it could be concluded that a T-independent response occurred at the small intestine level, where a clonal expansion of IgA+ B cells took place in the lamina propria. This would be possible considering that a recent report indicated that the B cell receptor can internalize soluble antigens and send immune signals of proliferation [43]. When B cells receptor (BCR) interact with antigens, transmembrane

signal transduction takes place, leading to modulation of gene expression, resulting in activation, anergy or apoptosis of B-cells [44]. The clonal expantion of the IgA+ cells would be favored by the enhanced levels of IL-6 found in the small intestine lamina propria due to the recognize participation of this cytokine in B cell terminal differentiation to plasmocytes [38]. Even we did not determine the role of Toll-like receptors in the immunomodulation exerted by kefiran, we think that TLR4 would be involved as was demonstrated for other polysaccharide antigen [45].

The effects observed on immunoglobulins and cytokine production in the large intestine after kefiran administration were more widely apparent than the ones observed at the small intestine lamina propria. Large communities of protein degrading and amino acid fermenting bacterial species exist in the colon, but, in numerical and metabolic terms, saccharolytic bacteria predominate in the microbiota [46]. Regarding the IgA+ cells number in the large intestine we could hypothesize that this increase was due to local stimulation or by stimulation in the intestinal lymph node. For the increase of IL-4+, IL-10+, IL-6+, IFNγ+ and TNFα+ cells, we suggest that this increase was due to a local stimulation in the large intestine, favored by the production of cytokines by cells from the innate immunity. But it is possible that T cells could also be involved in the production of cytokines measured.

The number of IgG+ cells in the small intestine did not differ from control mice in animals that received the exoplysaccharide. This would mean that no inflammatory immune response was induced by kefiran. The increased values of IgG+ cells in the large intestine when compared to control values could be not beneficial for the host because this fact may accelerate mucosal penetration of antigens and, as a consequence, a higher activation of the mucosal immune system. IgG may thus contribute to persistent immunopathology in mucosal disease [34]. However, the results of the histological studies (not showed) of the gut by haematoxylin–eosin stain showed no damage at the intestinal mucosal level when mice received kefiran even when there was a proliferation of IgG+ cells.

The increase in the numbers of IL-4+, IL10+, IL-6+ and TNFα+ cells in the large intestine for all feeding periods assessed, and for IFNα+ cells after day 7 of kefiran administration, may provide a form of "help" that is necessary for the induction and maintenance of a mucosal immune response (inflammatory and regulatory) at this levels. According to our results and taking in mind that polysaccharide antigens elicit a T-independent immune response, lead us believe that the increase in the number of cytokine+ cells observed in the large intestine lamina propria was due to an effect of kefiran mainly on cells of the innate immunity. However, we can not neglect the participation of the adaptive immunity that could have also been induced. Zwitterionic polysaccharides (ZPSs), those bearing both positively and negatively charged groups, have unique immunological properties: they can elicit potent CD4+ T cell responses in vitro, what distinguishes them from T-independent polysaccharides. ZPSs bind to the surface of antigen presenting cells, and the high density of charges facilitates electrostatic interactions with T cells [33,47].

In the present study, the effects of the oral administration of an exopolysaccharide on the cytokines induced were simultaneously compared in the small and large intestine and in the blood serum and intestinal fluid. The small intestine harbours the Peyer's patches that constitute the principal inductive sites of the immune response after the oral administration of an antigen. The small intestine immune system is anatomically connected to the systemic immune system by the lymphatic and blood circulation. The immune response induced in the small intestine can spread through the systemic immune system reaching mucosal and non-mucosal sites. The immune response induced in the large intestine is more confined to this environment. The greater immune response observed in the large intestine than in the small intestine after the exopolysaccharide administration shows a higher immune impact of the more degraded exopolysaccharide on the local immune cells of the large intestine and also due to an accumulation of circulating immune cells triggered through the inductive sites of the small intestine. We observed a smaller increase of cytokines in the intestinal fluid than in the blood serum, this could be due to the degradation by the luminal enzymes or fecal elimination. In blood serum, all the cytokines assayed followed a pattern of production that was quite similar to the one found for the same cytokines in the small intestine lamina propria, showing that even though we measured cytokine producing cells in the small intestine lamina propria, the cytokines were released into the circulation. This fact underline the importance of blood serum as a tool for the study of the immunomodulation by orally administered antigens in animal models.

This is a first report showing the importance of the polysaccharide produced by *L. kefiranofaciens* on the gut mucosa immunostimulation.

Acknowledgments

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