Administration of kefir-fermented milk protects mice against *Giardia intestinalis* infection

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Giardiasis, caused by the protozoan *Giardia intestinalis*, is one of the most common intestinal diseases worldwide and constitutes an important problem for the public health systems of various countries. Kefir is a probiotic drink obtained by fermenting milk with ‘kefir grains’, which consist mainly of bacteria and yeasts that coexist in a complex symbiotic association. In this work, we studied the ability of kefir to protect mice from *G. intestinalis* infection, and characterized the host immune response to this probiotic in the context of the intestinal infection. Six- to 8-week-old C75BL/6 mice were separated into four groups: controls, kefir mice (receiving 1 : 100 dilution of kefir in drinking water for 14 days), *Giardia* mice (infected orally with 4×10⁷ trophozoites of *G. intestinalis* at day 7) and *Giardia*–kefir mice (kefir-treated *G. intestinalis*-infected mice), and killed at 2 or 7 days post-infection. Kefir administration was able to significantly reduce the intensity of *Giardia* infection at 7 days post-infection. An increase in the percentage of CD4⁺ T cells at 2 days post-infection was observed in the Peyer’s patches (PP) of mice belonging to the *Giardia* group compared with the control and kefir groups, while the percentage of CD4⁺ T cells in PP in the *Giardia*–kefir group was similar to that of controls. At 2 days post-infection, a reduction in the percentage of B220-positive major histocompatibility complex class II medium cells in PP was observed in infected mice compared with the other groups. At 7 days post-infection, *Giardia*-infected mice showed a reduction in RcFce⁺-positive cells compared with the control group, suggesting a downregulation of the inflammatory response. However, the percentages of RcfCε-positive cells did not differ from controls in the kefir and *Giardia*–kefir groups. An increase in IgA-positive cells was observed in the lamina propria of the kefir group compared with controls at 2 days post-infection. Interestingly, the diminished number of IgA-positive cells registered in the *Giardia* group at 7 days post-infection was restored by kefir feeding, although the increase in IgA-positive cells was no longer observed in the kefir group at that time. No significant differences in CXCL10 expression were registered between groups, in concordance with the absence of inflammation in small-intestinal tissue. Interestingly, a slight reduction in CCL20 expression was observed in the *Giardia* group, suggesting that *G. intestinalis* might downregulate its expression as a way of evading the inflammatory immune response. On the other hand, a trend towards an increase in TNF-α expression was observed in the kefir group, while the *Giardia*–kefir group showed a significant increase in TNF-α expression. Moreover, kefir-receiving mice (kefir and *Giardia*–kefir groups) showed an increase in the expression of IFN-γ, the most relevant Th1 cytokine, at 2 days post-infection. Our results demonstrate that feeding mice with kefir reduces *G. intestinalis* infection and promotes the activation of different mechanisms of humoral and cellular immunity that are downregulated by parasitic infection, thus contributing to protection.

**INTRODUCTION**

Probiotics are defined as ‘live microorganisms which when administered in adequate amounts, exert a beneficial effect on host health’ (FAO/WHO, 2002). Through the years, the
food industry has released for public consumption a myriad of products containing live micro-organisms that are claimed to have health-promoting properties (Dekker et al., 2007). Probiotic micro-organisms can exert their beneficial actions through various different mechanisms, including competitive exclusion or inhibition of pathogens, modulation of the immune response, modification of intestinal microbiota and regulation of metabolic functions (Shah, 2007).

Kefir is a traditional fermented milk that originated many centuries ago in the Caucasus Mountains. It is obtained by fermentative activity of ‘kefir grains’. Kefir grains consist mainly of lactic acid bacteria (lactobacilli, lactococci, Leuconostoc), acetic acid bacteria and yeasts, which coexist in a complex symbiotic association in a protein–polysaccharide matrix (Garrote et al., 2001). Because several health-promoting properties, such as antimicrobial, antitumoral, hypocholesterolaemic and immunomodulating effects, have been associated with the consumption of kefir, this milk might be considered a probiotic product (Farnworth, 2005; Lopitz-Otsoa et al., 2006). However, there are few scientific reports of in vivo experiments supporting this empirical evidence (Thoreux & Schmucker, 2001; Vinderola et al., 2005, 2006; Lee et al., 2007; de Moreno de LeBlanc et al., 2007; Teruya et al., 2013), and many of the published reports are not available worldwide.

The use of in vivo models of infection to test the ability of different probiotic micro-organisms to protect against enteric pathogens has been extensively reported, mainly for bacterial infections. In contrast, their use in parasitic infections has been less studied (Benyacoub et al., 2005; Humen et al., 2005; Goyal et al., 2011, 2013; Travers et al., 2011).

Intestinal parasitic infections constitute an important problem for public health systems (WHO/PAHO, 1992). In particular, the zoonotic intestinal disease giardiasis, caused by the protozoan Giardia intestinalis, is one of the most common intestinal diseases worldwide. This parasite can infect children, adults and immunocompromised individuals, leading to acute or chronic diarrhoea, malabsorption, weight loss and growth delay in young children (Faubert, 2000; Cotton et al., 2011). Giardia infection is acquired by ingesting viable cysts as a result of inadequate sanitation or poor treatment of drinking water and contaminated foods (Amorós et al., 2010). Giardiasis can be treated with antibiotics such as nitroimidazoles and nitrofurans, but undesired side effects and emerging resistance have encouraged the search for alternative biotherapeutic strategies (Shukla et al., 2008; Shukla & Sidhu, 2011). Plant extracts, bee-derived products and probiotics are safe and inexpensive options with which to treat intestinal parasitosis (Calzada et al., 1999; Gardner & Hill, 2001; Travers et al., 2011). To date, however, there are few scientific reports on the effectiveness of probiotic micro-organisms for the treatment of giardiasis in animal models (Benyacoub et al., 2005; Humen et al., 2005; Shukla et al., 2008; Goyal et al., 2011, 2013) and no studies have involved the administration of fermented products.

Taking this into account, we decided to study the ability of kefir-fermented milk to protect mice from G. intestinalis infection, and to characterize the host immune response to this probiotic in the context of intestinal infection.

**METHODS**

**Parasite and culture conditions.** G. intestinalis strain H7 (ATCC 50581) was purchased from the American Type Culture Collection. Trophozoites were grown in TYI-S-33 medium supplemented with 10% bovine serum and antibiotic solution (1000 IU penicillin ml⁻¹ and 1 mg streptomycin ml⁻¹). The pH was adjusted to pH 6.9 with 5 N NaOH, prior to sterilization with a 0.22 μm (pore-size) filter. For experimental inoculation, actively growing trophozoites (48–72 h culture) were sedimented after chilling the tubes in ice for 10 min and were finally suspended in PBS (pH 7.2) to a final concentration of 2 × 10⁶ trophozoites ml⁻¹.

**Kefir culture.** Commercial UHT milk and kefir grains AGK1 from the CIDCA (Centro de Investigación y Desarrollo en Criotecnología de Alimentos) collection were used to obtain the fermented milk (Garrote et al., 2001). The granules were washed with water and incubated in milk in a proportion of 10% w/v for 24 h at 20 °C. The product obtained was filtered to separate the fermented milk from the granules, which were washed again and seeded in fresh milk. Kefir was prepared fresh every 24 h and was administered to mice diluted 1 : 100 in distilled water (the final concentration of bacteria and yeasts was approximately 10⁶ and 10⁴ c.f.u. ml⁻¹, respectively).

**Animals.** Female C57BL/6 mice aged 4–5 weeks were obtained from the Facultad de Ciencias Veterinarias (FCV) of the Universidad Nacional de La Plata and affirmed specific pathogen-free by the FCV’s Laboratory of Experimental Animals. Ten days prior to testing, the mice were housed under standard conditions in a 12 h light/dark cycle and were fed with a conventional diet and water ad libitum. All procedures were performed according to international guidelines for animal care and were approved by the FCV’s Committee for Use of Animals in Experiments.

**Experimental design.** Experimental animals were inoculated via intragastric gavage with 200 μl of a suspension containing 2 × 10⁶ trophozoites ml⁻¹ in sterile PBS. Control mice were inoculated with the same volume of sterile PBS. Animals were separated into four groups of 15 animals each.

In the Giardia group, mice were fed with milk (1 : 100) ad libitum for 6 days. On day 7, the mice were challenged orally with a single dose of Giardia trophozoites (4 × 10⁶ trophozoites per animal). Thereafter, milk feeding was continued until day 14.

In the Giardia–kefir group, mice were fed with kefir 1 : 100 ad libitum for 6 days. On day 7, a single dose of Giardia trophozoites (4 × 10⁶ trophozoites per animal) was given orally. Thereafter, kefir feeding was continued until day 14.

In the kefir group, mice were fed with kefir 1 : 100 ad libitum for 14 days.

In the control group, mice were fed with milk (1 : 100) ad libitum for 14 days.

All animals received food ad libitum. After 2 h, 2 days or 7 days post-infection, the animals were killed by CO₂ inhalation and cervical dislocation. Intestinal tissue, spleen, Peyer’s patches (PPs) and mesenteric lymph nodes (MLNs) were taken for analysis.
Counting of viable trophozoites. Mice belonging to the *Giardia* and the *Giardia*-kefir groups were killed and the proximal 10 cm section of the small intestine (mainly the jejunum) was removed and placed in 2 ml cold TYI-S-33 medium. To detach trophozoites, sections were washed with TYI-S-33 medium, opened longitudinally and homogenized in the same medium. The recovered trophozoites were counted using a haemocytometer.

Cell extraction from spleen, PPs and MLNs. The organs (spleen, MLN and PP) were removed aseptically and kept in sterile cold PBS until processing. Each organ was homogenized and washed with 5 ml sterile PBS. The obtained suspensions were centrifuged for 5 min at 400 g. In the spleen samples, red blood cells were lysed by suspending the pellet in 1 ml sterile water and immediately adding 9 ml RPMI 1640 medium (Invitrogen). The number of viable cells in each sample was determined by counting in a haemocytometer using Trypan blue. 400 μl of sterile PBS. The obtained suspensions were centrifuged for 5 min at 400 g. In the spleen samples, red blood cells were lysed by suspending the pellet in 1 ml sterile water and immediately adding 9 ml RPMI 1640 medium (Invitrogen). The number of viable cells in each sample was determined by counting in a haemocytometer using Trypan blue.

Labelling with specific antibodies for flow cytometry. Cells were labelled with the following antibodies: anti-mouse CD4 FITC-labelled, anti-mouse CD8 FITC-labelled, anti-mouse major histocompatibility complex (MHC) class II (I-A) PE-labelled, anti-mouse B220 FITC-labelled and anti-mouse RcFcε (all eBioscience). Each tube containing 3 x 10^6 cells was washed with FACS buffer (PBS with 1 % BSA) and incubated for 30 min on ice with the appropriately diluted primary antibodies. Unbound antibody was washed twice and, finally, labelled cells were fixed with 1 % paraformaldehyde in PBS. Isotype controls and unlabelled control cells were processed for each sample. Spleen cells labelled with anti-RcFcε were treated with FITC-conjugated streptavidin (eBioscience) for 30 min, washed twice with FACS buffer and fixed in 1 % paraformaldehyde in PBS.

Flow cytometry analysis. Cells were analysed by flow cytometry using FACS Caliber (Becton Dickinson). The differential cell count was determined after gating the lymphocyte population for forward- and side-scatter analysis.

Histological analysis. For histological analysis, 1 cm of the small intestine (collected 11 cm from the pylorus) was cut longitudinally, fixed in absolute ethanol and then dehydrated. Paraffin-embedded sections of 5 μm were rehydrated and stained with haematoxylin and eosin.

IgA-positive cell labelling. Histological slices were deparaffinized and rehydrated with xylene and 96 % ethanol. Samples were labelled with biotinylated anti-IgA 1:500 (eBioscience) for 30 min at 37 °C, washed twice with PBS and then added with streptavidin (STV)-FITC (Sigma) 1:250 for 20 min in darkness. Samples were then washed twice with PBS and examined using a fluorescent light microscope (Leica Microsystems) mounted in 50 % glycerol–PBS. Results are expressed as the number of IgA-positive cells per 10 fields.

Quantitative reverse transcription PCR (qRT-PCR). Samples of small intestine were homogenized and subjected to RNA extraction using the illustra RNA Spin Mini kit following the manufacturer’s instructions. Concentration was measured at 260 nm RNA. A total of 100 ng RNA reverse transcription using M-MLV reverse transcriptase (Promega) was performed and the resulting cDNA was amplified using Super IQ SYBR Green PCR Mix (Bio-Rad). The amplification reaction was carried out using an iCycler (Bio-Rad) according to the following protocol: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of amplification with 1 min annealing/extension at 60 °C and denaturation at 95 °C for 15 s. The expression of β-actin was used as a control. The specificity of the PCR was verified using melting curves. Primers for mouse CCL20, CXCL10, IL-12p40, TNF-α, IFN-γ and β-actin and relative difference calculations using the ΔCt method have been described previously (Rumbo et al., 2004; Anderle et al., 2005; Smaldini et al., 2012; Moreno et al., 2013).

Statistical analysis. Data were analysed using InfoStat software (Grupo InfoStat). Medians of trophozoite numbers and cell populations were compared using Mann–Whitney U tests. ANOVA and Student’s t-tests were used to compare results from other experiments.

RESULTS AND DISCUSSION

Effect of kefir administration on *G. intestinalis* infection

As shown in Fig. 1, the administration of kefir was able to reduce significantly the intensity of *Giardia* infection (number of viable trophozoites in intestinal lumen) at 7 days post-infection (medians 2.8 x 10^3 and 4.0 x 10^3 for the *Giardia* and the *Giardia*-kefir groups respectively; P<0.05), the time when maximal intensity of infection is usually observed in this and similar models (Benyacoub et al., 2005; Humen et al., 2005; Goyal et al., 2011). However, no significant differences in trophozoite counts were observed between the *Giardia* and *Giardia*-kefir groups at a shorter time (2 days) after infection (data not shown). Numerous studies have demonstrated the ability of probiotic micro-organisms to reduce parasitic protozoa infection. Humen et al. (2005) showed that administration of *Lactobacillus johnsonii* L1 significantly reduced the number of viable trophozoites in the intestine of gerbils (*Meriones unguiculatus*). Similar results were reported by Benyacoub et al. (2005) in a murine model with the administration of *Enterococcus faecium* SF68. Other authors have described that *Lactobacillus casei* MTCC 1423 was able to eliminate *Giardia* from infected mice, reducing atrophyed villi and infiltrating cells in small-intestinal tissue.
(Shukla et al., 2008). More recently, in vivo studies in malnourished mice showed that daily pre-treatment with *L. casei* MTCC 1423 reduced both the severity and the duration of giardiasis (Shukla & Sidhu, 2011). Regarding clinical trials, Besirbellioglu et al. (2006) showed that *Saccharomyces boulardii* reduced the number of parasite cysts in faeces from adult patients treated with a combination of *S. boulardii* and metronidazole versus patients treated only with metronidazole.

**Determination of immune cell populations by flow cytometry**

**T cells.** T cells are important markers of the response to *Giardia* infection (Singer & Nash, 2000), and we therefore evaluated CD4 and CD8 T-cell populations in immune inductive sites of intestinal mucosa (PP and MLN). A significant increase in the number of CD4⁺ T cells at 2 days post-infection was observed in PP of *Giardia*-infected mice (*Giardia* group) compared with the control and kefir groups (Fig. 2a; *P<0.05*), while no significant differences in CD4 T-cell number were observed in MLN (Fig. 2b). Mice belonging to the *Giardia*-kefir group had a similar percentage of CD4⁺ T cells in PP as the control and kefir groups. Although several authors have reported that the administration of probiotic bacteria can induce an increase in CD4⁺ T cells (Benyacoub et al., 2005; Vinderola et al., 2007; Goyal & Shukla, 2013), no changes were registered in other cases (Vitiñi et al., 2000; Galdeano & Perdigón, 2006; Humen, 2009). The latter is in agreement with our results and confirms the strain-specific nature of probiotic effects.

Percentages of CD8⁺ T cells (in PP and MLN) were similar among all the groups at 2 days post-infection (data not shown). In addition, no differences among the groups were observed after 7 days of infection (data not shown).

**Expression of MHC class II molecules in B cells.** The expression of MHC class II molecules in B cells (B220-positive cells) in PP and MLN was assessed as an activation marker. According to the level of MHC class II expression, B220-positive cells were subdivided into MHC class II low, medium and high populations (Fig. 3). A reduction in the percentage of B220-positive MHC class II medium cells in PP was observed in infected mice (*Giardia* group) compared with the other groups (*P<0.05*) at 2 days post-infection. No changes among the groups were registered for the MHC class II low and high populations (Fig. 3). These results suggest that *Giardia* infection may cause a decrease in the ability of B cells to process and present antigens to T cells, probably as a mechanism to evade the host’s adaptive immune response. Interestingly, activation is restored by kefir administration. Medrano et al. (2011) reported that kefiran (a polysaccharide extracted from kefir) induced an increase of B220-positive MHC class II high cells in the PP of BALB/c mice. These findings could indicate that kefiran, in part,
mediates the immune activation observed after kefir feeding. No differences between treatments were observed in B220-positive cells from MLN at either 2 or 7 days post-infection (data not shown).

**Mast cells (MCs).** MCs play a prominent role in the early immune response and are important for the rapid control of *Giardia* infection in mice (Li *et al.*, 2004). In this regard, it has been reported that MCs can contribute to the induction of specific IgA, since mice deficient in this cell population developed chronic giardiasis (Li *et al.*, 2004). In the current study, MCs (RcFcε-positive cells) at 2 and 7 days post-infection were quantified in the spleen. At 2 days post-infection, a significant decrease in MCs was observed in the kefir group, while values in infected animals (the *Giardia* and *Giardia*-kefir groups) were similar to those in the uninfected control group (Fig. 4a). In contrast, other researchers have reported an increase in mucosal MCs in mice fed with probiotics (Vinderola *et al.*, 2007).

At 7 days post-infection, *Giardia*-infected mice showed a reduction in MCs compared with uninfected controls, suggesting a downregulation of the inflammatory response. However, the percentages of MCs in kefir-treated animals (the kefir and *Giardia*-kefir groups) did not differ from the group control (Fig. 4b). We can hypothesize that feeding with kefir-fermented milk might restore the normal response, although further studies are needed to evaluate the influence of kefir on the mucosal MC population in order to test this hypothesis.

**Quantification of IgA-positive cells in lamina propria**

As it has been demonstrated that IgA is involved in the resolution of giardiasis (Langford *et al.*, 2002), the number of IgA-positive cells in the lamina propria was determined by immunofluorescence microscopy. Fig. 5(a) shows that at 2 days post-infection, an increase in IgA-positive cells (*P*<0.0001) was observed in the lamina propria of kefir-treated mice compared with control animals, suggesting that the increase in IgA-positive cells number was induced by kefir administration, which is in agreement with observations previously reported by Vinderola *et al.* (2005) in healthy mice after 2 and 5 days of treatment with kefir. Interestingly, the diminished number of IgA-positive cells

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**Fig. 4.** Percentage of RcFcε-positive cells (MCs) in the spleen at 2 days (a) and 7 days (b) following infection with *G. intestinalis*. C, uninfected control mice; G, *Giardia*-infected mice; GK, *Giardia*-infected mice treated with kefir; K, kefir-treated mice. *P*<0.05; **P**<0.01. Median values are shown inside the boxes. Error bars represent SD.

**Fig. 5.** Number of IgA-positive cells by immunofluorescence in the lamina propria of the small intestine after 2 days (a) and 7 days (b) post-infection. C, uninfected control mice; G, *Giardia*-infected mice; GK, *Giardia*-infected mice treated with kefir; K, kefir-treated mice. **P**<0.01; ***P***<0.0001. Median values are shown inside the boxes. Error bars represent SD.
seen in mice belonging to the *Giardia* group compared with controls (*P*<0.0001) at 7 days after infection was restored by kefir feeding (*P*<0.01 compared with the *Giardia* group), although the increase in IgA-positive cells was no longer observed in the kefir group at that time post-infection (Fig. 5b). Other authors have reported that a decrease in intestinal IgA is associated with more severe tissue damage (Faubert, 2000; Hawrelak, 2003). With this in mind, the increase in IgA-positive cells in the lamina propria could contribute to protection against *G. intestinalis* in our model.

**Determination of cytokines and chemokines in small-intestinal tissue**

In order to study the involvement of different molecules in the host immune response to giardiasis in the murine model, we evaluated the expression of chemokines (CCL20 and CXCL10) and cytokines (TNF-α, IL-12p40 and IFN-γ) by qRT-PCR in small-intestinal tissue.

Chemokines are molecules responsible for the chemotaxis of different cell populations and some have a key role in inflammatory responses. CCL20 is secreted by enterocytes in response to different inflammatory stimuli and can induce the migration of immature dendritic cells and lymphocytes (Ito et al., 2011). On the other hand, CXCL10 (also known as IP-10) is a pro-inflammatory mediator that mediates the attraction of different immune cell populations to inflamed or infected areas (Liu et al., 2011).

In our model, the expressions of CCL20 and CXCL10 were measured at two different times (2 h and 2 days) post-infection. Regarding CXCL10, similar levels of expression were observed in both infected (*Giardia* group) and uninfected control animals (*P*>0.05). However, a slight increase in CXCL10 expression was observed in kefir-treated mice (kefir and *Giardia*–kefir groups) compared with the *Giardia* group at 2 h post-infection, although no significant differences were found, which is in concordance with the absence of inflammation in the small-intestinal tissue of all the animals under study (data not shown). On the other hand, a slight reduction in CCL20 expression at 2 h post-infection was observed in infected mice (*Giardia* group) when compared with untreated, uninfected animals (control group) (Fig. 6a), suggesting that *G. intestinalis* might downregulate the expression of this chemokine as a way of evading the inflammatory immune response. Even though other authors have reported that infection of Caco-2 cells with *G. lamblia* induces a significant increase in CCL20 expression (Roxström-Lindquist et al., 2005), it is important to note that those experiments were performed with epithelial cell monolayers. According to our *in vivo* results, the mucosal cellular environment may condition the response of epithelial cells against the parasite.

Cytokines are small molecules that function as key immune mediators. As other investigators have described that *G. intestinalis* is able to negatively modulate the expression of early pro-inflammatory mediators in order to evade the innate immune response (Matowicka-Karna et al., 2009), we decided to analyse the expression of TNF-α. We did not observe differences between infected (*Giardia* group) and uninfected control mice (Fig. 6b), which is in agreement with previous results reported by Goyal & Shukla (2013) in a model of infection in BALB/c mice. However, a trend to an increase in TNF-α expression was observed in mice belonging to the kefir group. Fig. 6(b) shows that a significant increase in the expression of this cytokine was
registered at 2 h post-infection in kefir-treated infected mice (Giardia–kefir group). In concordance with our results, Vinderola et al. (2006) have reported an increase in the number of TNF-α-positive cells in the intestine even after 5 days of oral administration of kefir. Moreover, in our model, the increase in TNF-α expression was also observed in mice belonging to the Giardia–kefir group.

There were no significant differences in IL-12p40 expression between the groups under study at 2 h or 2 days post-infection (data not shown). In contrast, we observed an increase in the expression of IFN-γ, the most relevant Th1 cytokine, after 2 days of infection only in mice receiving kefir (kefir and Giardia–kefir groups) (Fig. 6c). These results suggest that administration of this probiotic fermented milk is responsible for the increase in IFN-γ expression in the small intestine, as was previously shown by Vinderola et al. (2005). As IFN-γ is a potent activator of macrophages and cytotoxic T cells, and taking into account that other authors have associated low levels of this cytokine with an increased susceptibility to Giardia infection (Faubert, 2000), we hypothesize that the increase in the expression of IFN-γ induced by kefir feeding could contribute to the protective effect of kefir against G. intestinalis infection.

Although there have been numerous reports on the ability of probiotics to protect against Giardia infection in animal models, none has evaluated the protective effect of a complex fermented product such as kefir milk. Our results demonstrate that feeding with kefir reduces G. intestinalis infection and promotes the activation of different mechanisms of humoral and cellular immunity that are down-regulated by parasitic infection, contributing to protection.

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