



The role of lactate on the immunomodulatory properties of the nonbacterial fraction of kefir



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ABSTRACT

The identification of components responsible for the bioactive properties of functional foods is of central interest in the food industry. In particular, fermented dairy products that are of a health benefit to the consumer may exert those salutary effects through the constituent microorganisms per se and/or through other bioactive components. Kefir is a beverage obtained by the fermentation of milk with kefir grains containing different lactic- and acetic-acid bacteria plus yeasts. We studied the immunomodulatory capacity of the nonbacterial fraction of kefir through approaches involving biochemistry and cell biology. Lactate, a major microbial metabolic product, was identified as the component responsible for the modulation of certain innate immune epithelial response: At the concentrations found in kefir-fermented milk, lactate inhibits the activation of intestinal epithelial cells triggered by interleukin-1 β , tumor necrosis factor- α , or flagellin. Lactate treatment furthermore abrogates NF- κ B signaling in the cells, whose action could be responsible for the observed modulation of the inflammatory response. These findings provide a new perspective in the analysis of the biologic properties of kefir-fermented-milk products.

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1. Introduction

Over the last decade, a major expansion of foods with health-promoting properties has taken place, giving rise to the so-called *functional foods*. This type of food, consumed as part of the normal daily diet, contains bioactive ingredients that offer health benefits including resistance to certain diseases. The identification of the bioactive ingredients is one of the principal objectives in the science of functional foods (Agyei & Danquah, 2012; Schwager, Mohajeri, Fowler, & Weber, 2008).

Consequently, the probiotics present within functional foods—and defined as living microorganisms that have been shown to exert beneficial effects on human health (FAO/WHO, 2002)—have been widely studied. Milk or milk products constitute excellent carriers for these probiotics wherein specific bacterial-fermentation processes may lead to milk products with new physiologic effects beyond the normal

nutritional function of the dairy product alone. In addition to alterations in texture and flavor and improvement in digestibility, the fermentation of milk can also serve to create, enrich, or release new milk-associated functional components (Beermann & Hartung, 2012). Enzymatic bio-transformations—such as glycolysis, proteolysis, and lipolysis as well as the synthesis of organic acids and ethanol—are the primary reactions of milk fermentation. In order to exert their associated health benefits, probiotic microorganisms need to be alive, though some of those effects could be achieved by soluble products elaborated by those microbes or their lysates (Kverka et al., 2009). Milk fermentation by lactic-acid bacteria leads to the release of bioactive peptides from milk proteins (Beermann & Hartung, 2012; Jakala & Vapaatalo, 2010), an enrichment of essential vitamins (Hugenholtz, Hunik, Santos, & Smid, 2002), and/or the liberation of oligo- or polysaccharides with biologic activity (Ruas-Madiedo, Abraham, Mozzi, & De Los Reyes-Gavilán, 2008). The immunomodulatory effects of fermented milks involving potential benefits to human health and reductions in disease risk have also been well documented. In this regard, numerous studies have demonstrated that the lactic-acid bacteria in fermented milk enhance specific and/or non-specific immune response (Isolauri, Salminen, & Ouwehand, 2004; Matar, Valdez, Medina, Rachid, & Perdigon, 2001; Tsai, Cheng, & Pan, 2012).

Kefir is a traditional beverage obtained by the fermentation of milk with kefir grains containing a wide diversity of lactic- and acetic-acid bacteria plus yeasts (Ahmed et al., 2013; Garrote, Abraham, & De Antoni,

Abbreviations: AGS, gastric-epithelial cell line; CCL20, chemokine ligand 20; CXCL2, chemokine ligand 2; CXCL10, chemokine ligand 10; DMEM, Dulbecco's Modified Eagle's Minimum Essential Medium; FltC, flagellin; GPR, G-protein-coupled receptor; IL-1 β , interleukin-1 β ; IL-8, interleukin-8; ITF, intestinal-trefoil factor; LDH, lactate dehydrogenase; LPH, lactase-phlorizin hydrolase; MIF, macrophage-migration-inhibitory factor; MSAA, milk supernatant artificially acidified; SCFA, short-chain fatty acids; TNF- α , tumor-necrosis factor α .

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2010). Beyond the drink's inherent high nutritional value as a source of proteins and calcium, kefir is considered a functional food. The health-promoting properties of kefir have been widely proven (Ahmed et al., 2013; Garrote et al., 2010; Guzel-Seydim, Kok-Tas, Greene, & Seydim, 2011), including a reduction in lactose intolerance (De Vrese, Keller, & Barth, 1992; Hertzler & Clancy, 2003), a lowering of blood-cholesterol levels (Liu et al., 2006), antimutagenic and anticarcinogenic properties (De Moreno de LeBlanc, Matar, Farnworth, & Perdígón, 2007; Liu, Wang, Lin, & Lin, 2002), antagonism against pathogens (Golowczyk, Mobili, Garrote, Abraham, & De Antoni, 2007), antimicrobial activity (Garrote, Abraham, & De Antoni, 2000; Londero et al., 2011), and a stimulation of the immune system (Romanin et al., 2010; Vinderola et al., 2005; Vinderola, Perdígón, Duarte, Thangavela et al., 2006). The health benefits associated with kefir consumption may be exerted by the presence of the microorganisms themselves and/or by other bioactive components. A study of the nonbacterial fraction of this fermentate is accordingly essential in gaining a greater understanding of kefir's inherent biologic activity. The present investigation was therefore undertaken in an attempt to analyze the bioactive properties of the nonbacterial fraction of kefir-fermented milk with a focus on the ability to modulate the innate immune response of intestinal-epithelial cells.

2. Materials and methods

2.1. Kefir grains and milk fermentation

Kefir grains CIDCA AGK1 and CIDCA AGK10 belonging to the collection of the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, La Plata, Argentina) were added to skim milk at a concentration of 10% (w/v). Fermentation was conducted for 24 h at 20 °C. In some experiments milk fermented for 48 h was also studied. The kefir grains were then separated from the fermentate by filtration through a plastic sieve. The fermentation products were centrifuged and the supernatants were neutralized and filtered through a 0.45- μ m membrane (to obtain the nonbacterial fraction). In addition, an aliquant (1 mL) of that nonbacterial fraction was heated in a water bath at 100 °C for 15 min (the heated fraction) and another portion (5 mL) was dialyzed against distilled water at 4 °C for 48 h (the dialyzed fraction) through the use of a cellulose membrane of molecular-weight cut-off of 1000 Da (Spectra/Por® 7, Spectrum Laboratories Inc., USA). All fractions were maintained at –20 °C until use. The pH of the fermented products was measured with a Model pH 211 pH meter equipped with an HI 1330B microelectrode (Hanna Instruments, USA). The organic acids present were characterized both qualitatively and quantitatively by high-performance liquid chromatography as previously described (Garrote et al., 2000). Artificial milk supernatants and acid-water solutions were prepared by the addition of racemic D,L-lactic acid (J.T. Baker, USA) and acetic acid (Dorwil, Argentina) at the concentrations found in fermented milk and then neutralized and filtered as described above.

2.2. Epithelial-cell lines and reagents

The human epithelial colorectal-adenocarcinoma-cell line Caco-2 was a gift from Dr. J.C. Sirard (Institut Pasteur, Lille, France). The epithelial human-stomach-adenocarcinoma-cell line (AGS) was a kind gift from Dr. H. De Reuse (Institut Pasteur, Paris, France). Caco-2 cells stably transfected with a luciferase reporter construction under the control of the chemokine-ligand-20 (CCL20) promoter (Caco-2 ccl20:luc) have been previously described (Nempont et al., 2008). The cells were routinely grown in Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM, GIBCO BRL Life Technologies, Rockville, MD, USA); supplemented with 15% (v/v) heat-inactivated (30 min, 60 °C) fetal-bovine serum (FBS, PAA, GE Healthcare Bio-Sciences Corp., USA), 1% (v/v) nonessential amino acids (GIBCO BRL Life Technologies, Rockville, MD, USA), and the following antibiotics (Parafarm, Saporiti SACIFIA,

Buenos Aires, Argentina): penicillin (12 IU/mL), streptomycin (12 μ g/mL), and gentamicin (50 μ g/mL). Caco-2-ccl20:luc cells were used at 24 h postconfluence after 8 days of culture at subculture passages between 12 and 22 from the original stocks. All experiments were performed in serum-free medium.

For the analysis of human tissue, patients who had undergone an intestinal-transplantation operation at the Favaloro Foundation University Hospital (Buenos Aires, Argentina) were enrolled. Tissue specimens from those patients were obtained either from tissue biopsies removed during routine endoscopic surveillance early after the surgery (from either the small- or large-intestinal mucosa) or from the ileostomy-closure operation (from visceral adipose tissue). None of the samples obtained were from infected, inflamed, or neoplastic tissues. The present protocol was approved by the Institutional Review Board and Ethics Committee of Favaloro Foundation (DDI (984) 1207). Informed consent was obtained in all cases.

Flagellin (FliC)—obtained and purified from *Salmonella enterica* serovar Enteritidis as previously described (Sierro et al., 2001)—was used as an inducer of the proinflammatory response. Other proinflammatory stimulators, such as human interleukin-1 β (IL-1 β) and tumor-necrosis factor α (TNF- α) were purchased from R&D Systems (Minneapolis, MN, USA).

2.3. Stimulation assay with the Caco-2-ccl20:luc reporter system

Confluent Caco-2-ccl20:luc cells cultured in 48-well plates were treated for 30 min with fermented products, artificially acidified milk supernatants, or either aqueous solutions of racemic lactic acid or acetic acid or a mixture of both diluted in serum-free DMEM (1:1 [v/v]). The cells were then exposed to stimulation by FliC (1 μ g/mL), IL-1 β (10 ng/mL) or TNF- α (100 ng/mL) during a 6-h incubation at 37 °C in an atmosphere of 5% CO₂–95% air. A basal condition without any treatment was included as a control lacking stimulation; while FliC, IL-1 β , or TNF- α respectively was added as controls for conditions producing a 100% induction of the proinflammatory response. The cells were next lysed with Lysis Buffer (Promega, Madison WI, USA) and luciferase activity was measured as previously described (Nempont et al., 2008). Luminescence was normalized to the stimulated control cells and expressed as a percentage of the normalized average luminescence \pm standard deviation (SD) from at least three independent experiments.

After the above treatments, the membrane integrity of Caco-2-ccl20:luc cells, grown in 6-well plates, was evaluated by measuring the lactate-dehydrogenase (LDH) activity with the LDH-P Unitest Kit (Weiner Lab, Rosario, Argentina) according to the manufacturer's instructions. The results were expressed as the percent LDH activity in the culture medium relative to total LDH activity (released after lysing the cells). As a method of assessing treatment-induced cytotoxicity, mitochondrial activity was evaluated by measuring the mitochondrial-dependent reduction of colorless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, USA) to a purple-colored formazan (Mosmann, 1983) as previously described by Medrano, Perez, and Abraham (2008).

2.4. Transient transfection of Caco-2 cells and stimulation assay

Caco-2 cells were transfected through the use of Lipofectamine™ 2000 (Invitrogen, USA) with plasmids containing *Renilla* spp. luciferase under the control of the HSTK promoter and firefly luciferase under a NF- κ B-dependent promoter (3X- κ B artificial promoter) as previously described by Romanin et al. (2010). Stated in brief, transfected cells were preincubated with a 100 mM lactic-acid solution at pH 7.0 and then stimulated with FliC as described above. After treatment, luminescence was measured with the Dual Luciferase Assay Kit (Promega, USA) after the manufacturer's instructions.

2.5. In vitro analysis of chemokine expression

Cell monolayers of Caco-2 ccl20:luc in 24-well cell-culture plates were incubated for 30 min with 500 μ L of aqueous lactic-acid solutions that had been previously neutralized and filtered as described above. The cells were then stimulated with FlIC (1 μ g/mL). Two hours after stimulation, the samples were homogenized in RA1 Lysis Buffer (GE Healthcare, UK) to extract the total RNA as described below.

2.6. Determination of gene expression in cell lines and tissues

For the determination of gene expression in different cell lines and tissues, cDNA from AGS gastric-epithelial-carcinoma cells, Caco-2 cells, human-adipose tissue, and human-colonic and small-bowel tissue was prepared by total-RNA extraction from the cell cultures or the tissues through the use of the RNeasy Spin Kit (GE Healthcare, UK) followed by a reverse transcription from random primers (Invitrogen, USA), as previously described by Errea et al. (2010).

2.7. Real-time polymerase-chain-reaction (PCR) analysis

Total RNA was extracted by means of the Illustra RNeasy Mini RNA Isolation Kit (GE Healthcare, UK). Reverse transcription was performed from random primers and MMLV reverse transcriptase (Invitrogen, USA). Real-time PCR was carried out according to the manufacturer's protocol through the use of an iCycler thermal cycler (BioRad, USA).

The primers for CCL20, interleukin-8 (IL-8), chemokine ligand 2 (CXCL2), chemokine ligand 10 (CXCL10), lactase-phlorizin hydrolase (LPH), and human β actin along with the relative-difference calculation by means of the Δ Ct method have been previously described (Anderle et al., 2005; Rumbo, Sierro, Debar, Kraehenbuhl, & Finke, 2004). The intestinal-trefoil factor (ITF) and macrophage-migration-inhibitory factor (MIF) were assayed as described by Romanin et al. (2010); while expression of the gene encoding the G-protein-coupled receptor 81 (GPR81) in Caco-2-ccl20:luc, AGS, and human-small-intestine and colon cells was detected through the use of the primers described by Liu et al. (2009).

2.8. Statistical analysis

Differences in luciferase activity and mRNA-expression levels were tested statistically by the one-way ANOVA and Tukey's multiple-comparison test conducted by the GraphPad Prism® software to determine any significant differences.

3. Results

3.1. Downregulation of the Caco-2-ccl20:luc reporter by the nonbacterial fraction of kefir

We studied the effects of the nonbacterial fraction from kefir on the reporter system Caco-2 ccl20:luc. This system consists of intestinal Caco-2 cells stably transfected with a luciferase-reporter construction under the control of the CCL20 promoter (Nempont et al., 2008). In response to stimulation with FlIC the transfected cells are induced to express CCL20 and consequently manifest a high luciferase activity. Preincubation of these luciferase-expressing cells for 30 min with the nonbacterial fraction from a milk fermented for 24 or 48 h with kefir grains from different origins produced an 80% inhibition of luciferase activity (Fig. 1A). The downregulation was maintained after heating, but was lost upon dialysis of the fermentate (Fig. 1B), indicating that a low-molecular-weight component could be responsible for the inhibition. Neither the integrity nor the viability of the cultured cells was affected by treatments with the nonbacterial fraction from kefir fermentates since the percent LDH activity in the culture medium was not significantly different from that of the control cells ($p < 0.05$). Moreover, the mitochondrial activity of the treated cells was likewise not significantly altered ($p < 0.05$). A similar downregulation by the nonbacterial fraction from kefir fermentates was also observed when the Caco-2-ccl20:luc cells were stimulated with the proinflammatory agents IL-1 β or TNF- α (Fig. 2A and B).

As the nonbacterial fraction of kefir obtained with CIDCA AGK1 and CIDCA AGK10 grains contained lactic and acetic acids as the main fermentation products (Table 1), we assayed milk supernatants that had been artificially acidified with the organic-acid concentrations present in the fermented milk or aqueous solutions of either lactic acid (100 mM, pH 7.0) or acetic acid (5 mM, pH 7.0) under the same conditions. The lactic-acid solution or the artificially acidified milk supernatant was capable of inhibiting luciferase activity to a degree comparable to that of the nonbacterial fraction of kefir, while the solution of acetic acid produced no such modulation (Fig. 3). A comparable behavior was observed independently with the proinflammatory-stimulating agents employed (data not shown).

We performed a dose-response assay using the two principal organic acids present in kefir and observed a correlation between their concentration and the capacity to modulate the proinflammatory response (Fig. 4).

Since the concentration of acetate found in our fermented product was <12 mM (Table 1), the contribution of that organic acid to the observed activity can only be minor; so that the lactate present must

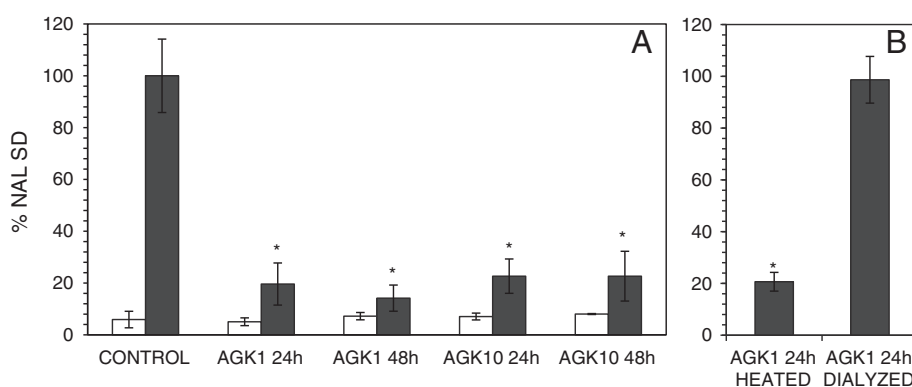


Fig. 1. Nonbacterial fraction of kefir modulates innate immune activation triggered by flagellin in intestinal-epithelial cells. (A) Percent normalized average luminescence (%NAL) of Caco-2-ccl20:luc cells either untreated (CONTROL) or preincubated with nonbacterial fractions from different kefir-fermented milks without stimulation (white bars) or stimulated with FlIC (black bars). (B) %NAL of Caco-2-ccl20:luc cells first preincubated with either the heated or the dialyzed nonbacterial fraction of milk fermented with CIDCA AGK1 kefir grains for 24 h and then exposed to stimulation by FlIC.*Indicates a significant difference from the value for the FlIC-stimulated control cells ($p < 0.05$; Tukey's multiple comparison test). SD: standard deviation.

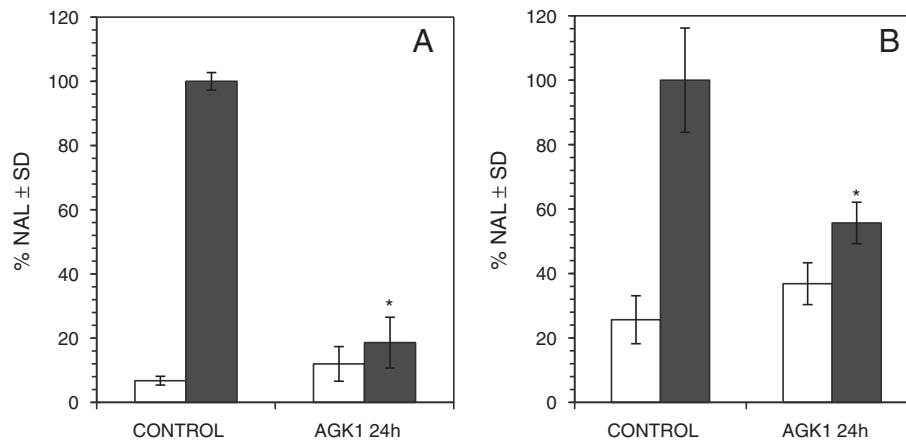


Fig. 2. Nonbacterial fraction of kefir modulates inflammatory activation of epithelial cells triggered by different proinflammatory stimuli. Percent normalized average luminescence (% NAL) of Caco-2-ccl20:luc cells either untreated (CONTROL) or preincubated with the nonbacterial fraction of milk fermented with CIDCA AGK1 kefir grains for 24 h either without stimulation (white bars) or stimulated (black bars) with IL-1 β (A) or TNF- α (B). *Indicates a significant difference from the value for the stimulated control cells ($p < 0.05$; Tukey's multiple-comparison test). SD: standard deviation.

be the main component responsible for modulating the activation of innate response.

3.2. Lactate preincubation modulates NF- κ B signaling activation

The activity of the CCL20 promoter may rely on different signaling-pathway activations. In order to analyze the effects exclusively on the NF- κ B pathway, we used cells harboring a transient transfection with a reporter driven by an artificial promoter including tandem 3- κ B binding sites in control of luciferase activity. In this culture system, a preincubation with lactate abolished the activation of NF- κ B induced by FliC (Fig. 5).

3.3. Lactate preincubation down-modulates different effectors of epithelial proinflammatory response without affecting other enterocyte functions

We also analyzed the effect of lactate at the transcriptional level using real-time quantitative PCR and observed that the expression of the genes for several chemokines and/or cytokines (e.g., CCL20, CXCL2, CXCL10, and IL-8) involved in the inflammatory response was not affected by pretreatment with lactate, whereas the transcription of those genes increased significantly when stimulated with FliC. Furthermore, exposure to lactate produced a significant downregulation of the expression of those inflammation-mediating genes (Fig. 6A); whereas the same lactate pretreatment had no effect on the transcription of genes involved in normal enterocyte function—e.g., LPH, ITF, and MIF—thus indicating a specific inhibition by lactic acid of those proinflammatory pathways (Fig. 6B).

These results in combination indicate a general anti-inflammatory effect on intestinal epithelium produced by preincubation with lactic acid.

3.4. Detection of specific receptors in intestinal-epithelial cells

Considering lactate's immunomodulatory effects and that this organic acid had been identified as a ligand for the G-protein-coupled receptor

GPR81 expressed in adipose tissue (Hongfei et al., 2008), we studied the expression of this receptor gene in the intestinal-epithelial line Caco-2 ccl20:luc, in the gastric-epithelial AGS cells, and in epithelial cells from human small-bowel tissue and colon; with human adipose tissue being used as a positive control. A real-time PCR reaction was conducted, and the results were normalized with respect to β -actin expression (Fig. 7). Adipose tissue exhibited the highest expression of the receptor gene, whereas GPR81-specific-mRNA transcription was also clearly detected in the Caco-2 cells as well as in the samples from the small bowel or the colonic mucosa from 5 different patients. GPR81-mRNA expression, however, was lower in the gastric-epithelial cell line.

4. Discussion

In the present investigation we characterized the ability of the non-bacterial fraction of kefir to modulate the activation of epithelial innate inflammatory responses and unexpectedly found that a major contributor to this property was lactate. A principal fermentation metabolite of the constituent lactic-acid bacteria in kefir, lactate, is an organic acid that can be found as a component of different fermented products and is also generated in situ by the microbiota of the intestinal lumen. The contribution to this modulation by other soluble compounds also present in the nonbacterial fraction of kefir, however, could not be ruled out. In recent years, short-chain fatty acids (SCFA) have been recognized as a major environmental signal in intestinal mucosa with the ability to stimulate the regulatory T-cell compartment (Arpaia et al., 2013; Smith et al., 2013) as well as nonimmune mucosal cells (Nøhr et al., 2013). Most of these activities have been attributed to butyrate, acetate, or propionate; but, to the best of our knowledge, the present report is the first showing the role of lactate in regulating inflammatory functions in intestinal-epithelial cells.

The production of lactate is a hallmark feature of the fermentation of lactic-acid bacteria, and kefir is a fermented milk containing one of the highest levels of this acid (ca. 100 mM; Table 1), probably as a result of the combined action of the kefir-grain microbial consortium (Garrote et al., 2010). The immunomodulatory properties of kefir have been

Table 1
Organic-acid concentrations and pH after fermentation of milk with kefir grains CIDCA AGK1 and CIDCA AGK10 for 24 and 48 h.

Kefir grain	Fermentation time (h)	pH	Lactic acid (mM)	Acetic acid (mM)
CIDCA AGK1	24	4.02 \pm 0.21	90.5 \pm 6.3	4.3 \pm 1.5
	48	3.65 \pm 0.32	129.7 \pm 15.3	9.8 \pm 2.2
CIDCA AGK10	24	4.32 \pm 0.10	86.2 \pm 9.1	4.6 \pm 2.7
	48	3.70 \pm 0.12	115.4 \pm 8.4	11.3 \pm 5.9

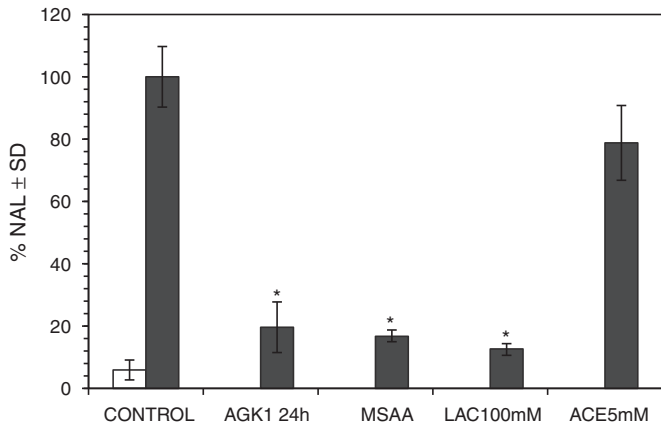


Fig. 3. Lactate exhibits modulatory capacity for epithelial innate activation comparable to nonbacterial fraction of kefir. Percent normalized average luminescence (% NAL) of Caco-2-ccl20:luc cells either untreated (CONTROL) or preincubated with either the nonbacterial fraction of milk fermented with CIDCA AGK1 kefir grains for 24 h (AGK1 24 h), milk supernatant artificially acidified with organic acids (MSAA), a 100 mM lactic-acid solution at pH 7 (LAC 100 mM), or a 5 mM acetic-acid solution at pH 7 (ACE 5 mM) and then either left without stimulation (white bars) or stimulated with FliC (black bars). *Indicates a significant difference from the value for the FliC-stimulated control cells ($p < 0.05$; Tukey's multiple-comparison test). SD: standard deviation.

demonstrated in vivo (Vinderola et al., 2005; Vinderola, Perdígón, Duarte, Farnworth, & Matar, 2006). Kefir, pasteurized kefir, and the products derived from milk fermentation by kefir microflora have been shown to induce a mucosal response in which different fractions were able to promote a protective immunity, maintain the intestinal homeostasis, and

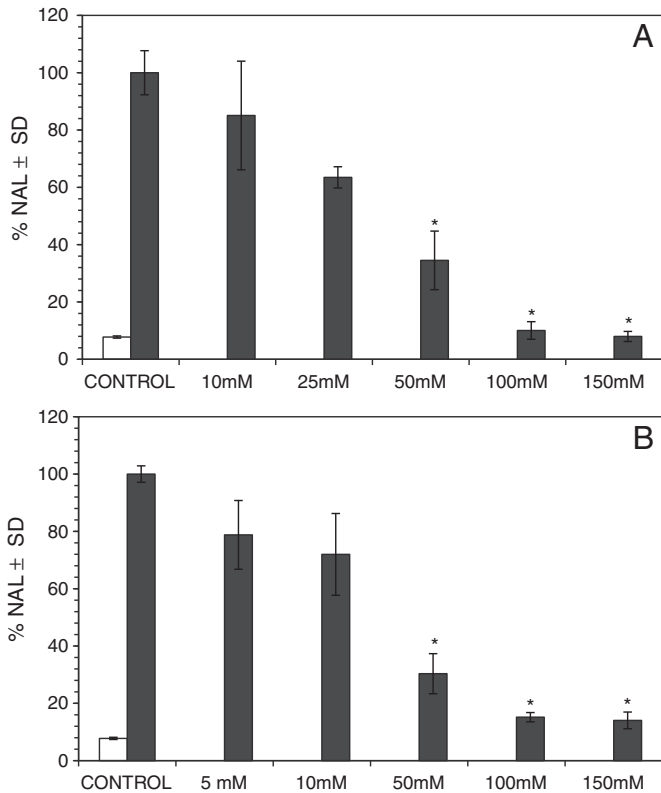


Fig. 4. Dose–response profile for activation modulation by the two principal organic acids present in kefir. Percent normalized average luminescence (% NAL) of Caco-2-ccl20:luc cells either untreated (CONTROL) or preincubated with different concentrations of aqueous lactic (A) or acetic (B) acid solutions at pH 7 and then either left without stimulation (white bars) or stimulated with FliC (black bars). *Indicates significant difference from the value for the FliC-stimulated control cells ($p < 0.05$; Tukey's multiple-comparison test). SD: standard deviation.

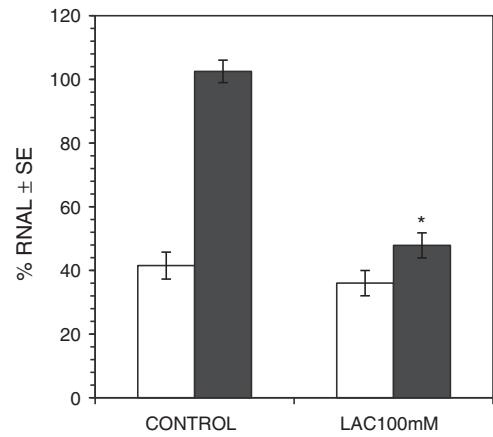


Fig. 5. Modulation of NF- κ B activation by lactate. Percent relative normalized average luminescence (% RNAL) of Caco-2 cells transiently transfected with 3X- κ B:luc and HSV-TK:Renilla luciferase reporter, then either untreated (CONTROL) or preincubated with a 100 mM aqueous lactic-acid solution pH 7, and finally either left without stimulation (white bars) or stimulated with FliC (black bars). *Indicates significant difference from the value for the FliC-stimulated control cells ($p < 0.05$; Tukey's multiple-comparison test). SE: standard error.

enhance immunoglobulin A production in both the small and the large intestine. In particular, kefir consumption was observed to have a more pronounced effect than a direct administration of the kefir-associated bacteria (Vinderola, Perdígón, Duarte, Farnworth et al., 2006). The lactic acid produced by probiotic lactobacilli has been recently shown to be potentially critical for the modulation of inflammation in an indomethacin-induced injury of the small intestine (Watanabe et al., 2009). In their

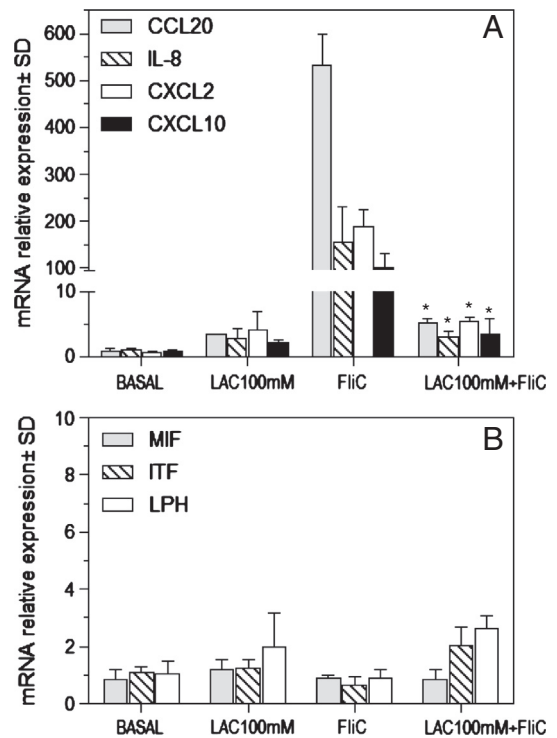


Fig. 6. Modulation by lactate of the expression of several proinflammatory cytokines without affecting normal enterocyte functions. Relative expression of mRNA encoding proinflammatory chemokines and/or cytokine genes (A) and genes involved in normal enterocyte function (B) of Caco-2-ccl20:luc cells under basal conditions (BASAL), treated with 100 mM lactic-acid solution pH 7 (LAC 100 mM), nontreated and stimulated with FliC (FliC), and treated with 100 mM lactic-acid solution pH 7 and stimulated with FliC (LAC 100 mM + FliC). *Indicates significant difference from the value for the FliC-stimulated control cells ($p < 0.05$; Tukey's multiple-comparison test).

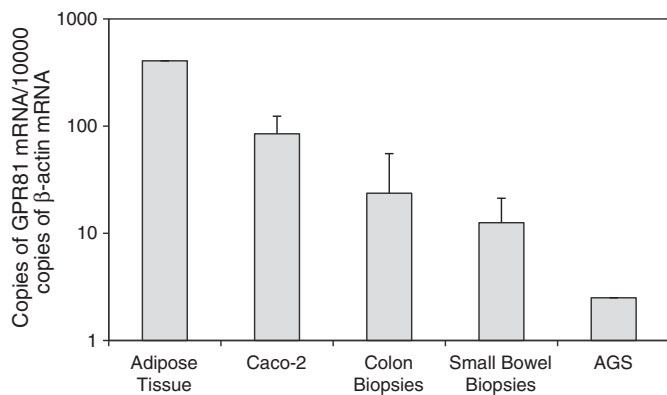


Fig. 7. Lactate receptor (GPR81) is expressed in intestinal epithelial cells and human intestinal mucosa. Copies of GPR81 mRNA per 10,000 copies of β -actin mRNA in adipose tissue, Caco-2 cells, colon biopsies, small-bowel biopsies and gastric-cell line (AGS).

work they also demonstrated the ability of lactate to modulate NF- κ B signaling in myeloid cells. Part of the protective effects of that acid described by Watanabe et al. (2009) could possibly be a result of the action of lactate on intestinal-epithelial cells as shown here. Furthermore, other bioactive properties of lactate on intestine have been recently described by Okada et al. (2013), who showed that luminal lactate stimulates enterocyte proliferation in a starvation–re-feeding murine model. It is possible that some of the immunomodulating properties of kefir consumption shown in vivo may be related to the particularly high levels of lactate found in this fermented milk.

According to the dose–response profile we obtained (Fig. 3), lactate down-regulates the activation of innate responses at concentrations around 100 mM, which levels correspond to the range produced in kefir-fermented milk (Table 1). Since lactic acid can be found in other fermented foods at levels from about 5 to 100 mM (Fernández Villamil, Miranda Ordieres, & Tuñón Blanco, 1997; Shapiro & Silanikove, 2010), those fermentates should be able to exert the biologic activity shown here to different degrees. Although lactate can be generated by the intestinal microbiota, the concentration that is typically achieved in the colon—at from around 10 to 30 mM—is lower than that in the fermented milk (Thompson–Chagoyan et al., 2011). Possible rises in lactate concentration under certain pathophysiologic situations, such as intestinal ischemia or sepsis, may also reflect an endogenous production of lactate by the host (Jorgensen, Reiter, & Perner, 2006; Solligard et al., 2008). Consequently, although lactate may be generated in situ in the intestinal lumen, the ingestion of fermented-milk products may constitute a convenient source of lactate at concentrations high enough to enable a triggering of modulatory processes like those described here.

We observed that lactate down-regulates the expression of several proinflammatory molecules in enterocytes without affecting the expression of enterocyte function (Fig. 6). This modulatory capacity of lactate was observed upon activation of enterocytes by different proinflammatory stimuli (Figs. 1 and 2), thus indicating a general mechanism of action within the inflammation process. Consistent with that notion, we confirmed that the activation of NF- κ B signaling, a master regulator of the proinflammatory response (Baltimore, 2011), was abrogated by lactate preincubation (Fig. 5), in coincidence with previous results obtained with the THP-1 myeloid cell line (Watanabe et al., 2009). Although the identification of the mechanism of action of lactate at the molecular level was not one of the objectives of the present work, lactate may conceivably trigger some specific form of signaling in enterocytes that mediate its effects. In recent years, several receptors with the ability to bind SCFA or related metabolites and to control several physiologic functions have been described. The G-protein-coupled receptors GPR43 (or FFAR2) and GPR41 (or FFAR3) are involved in the salutary effects of SCFA on appetite, energy metabolism, and the regulation of

immune function (Viñolo, Rodrigues, Nachbar, & Curi, 2011). In-vitro studies have shown that acetate preferentially activates GPR43, propionate GPR43 and GPR41, and butyrate GPR41 (Maslowski et al., 2009; Schmidt et al., 2011). Likewise described in the literature are specific receptors for lactate such as GPR81 (or HCA1) that is expressed predominantly in adipocytes and has an antilipolytic effect (Hongfei et al., 2008; Liu et al., 2009). The related receptor of the present family, GPR109A, whose protein can bind 2-hydroxybutyrate and butyrate, mediates anti-inflammatory functions on several cell types and is expressed in intestinal epithelium (Offermanns, 2013). In this investigation we have shown that GPR81 is also expressed in human intestinal-epithelial cells and intestinal-mucosal tissue (Fig. 7), thus making that receptor an attractive candidate for mediation of the anti-inflammatory effects of lactate observed in the work reported here.

5. Conclusions

The results obtained in this study have demonstrated that the non-bacterial fraction obtained from milk fermented with kefir grains has a significant capacity to down-regulate the intestinal-epithelial innate inflammatory responses in culture. Using biochemical and cell-biology approaches, we were able to identify lactate as a major component having immunomodulatory properties in intestinal-epithelial cells. Moreover, the expression of a specific lactate receptor in those cells has been described in this report for the first time. These findings provide a new perspective in the analysis of the biologic properties of fermented products by lactic acid bacteria.

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