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

Carolina Iraporda [a], David E. Romanin [b], Martín Rumbo [b], Graciela L. Garrote [a,*], Analía G. Abraham [a,c]

[a] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, UNLP-CONICET), Calle 47 y 116, 1900 La Plata, Argentina
[b] Laboratorio de Investigaciones del Sistema Inmune (LISIN, UNLP), Calle 47 y 115, 1900 La Plata, Argentina
[c] Área Bioquímica y Control de Alimentos, Facultad de Ciencias Exactas, UNLP, Calle 47 y 115, 1900 La Plata, Argentina

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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-1β, tumor necrosis factor-α, or flagellin. Lactate treatment furthermore abrogates NF-κB 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.



## 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 biotransformations—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 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, & Perdigón, 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; FliC, flagellin; GPR, G-protein-coupled receptor; IL-1β, interleukin-1β; 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.

* Corresponding author. Tel.: +54 221 4254853; fax: +54 221 4249287.
*E-mail address:* ggarrote@biol.unlp.edu.ar (G.L. Garrote).

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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, & Perdigón, 2007; Liu, Wang, Lin, & Lin, 2002), antagonism against pathogens (Golowczyc, 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, Perdigón, 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-μm 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 μg/mL), and gentamicin (50 μg/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-1β (IL-1β) 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 μg/mL), IL-1β (10 ng/mL) or TNF-α (100 ng/mL) during a 6-h incubation at 37 °C in an atmosphere of 5% $CO_2$–95% air. A basal condition without any treatment was included as a control lacking stimulation; while FliC, IL-1β, 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 ± 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-κB-dependent promoter (3X-κB 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.