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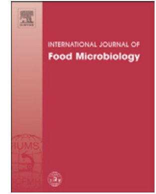

Down-regulation of intestinal epithelial innate response by probiotic yeasts isolated from kefir

David Romanin [a,b], María Serradell [b], Dolores González Maciel [a], Natalia Lausada [c], Graciela L. Garrote [b,d], Martín Rumbo [a,*]

[a] Laboratorio de Investigaciones en el Sistema Inmune (LISIN), FCE, UNLP, 47 y 115, La Plata, Argentina
[b] Cátedra de Microbiología, FCE, UNLP. 47 y 115, La Plata, Argentina
[c] Laboratorio-Programa de Transplante de Organos y Tejidos, FCM, UNLP, 60 y 120, La Plata, Argentina
[d] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) CCT La Plata CONICET, 47 y 116, La Plata, Argentina

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ABSTRACT

Kefir is obtained by milk fermentation with a complex microbial population included in a matrix of polysaccharide and proteins. Several health-promoting activities has been attributed to kefir consumption. The aim of this study was to select microorganisms from kefir able to down-regulate intestinal epithelial innate response and further characterize this activity.

Caco-2 cells stably transfected with a human CCL20 promoter luciferase reporter were used to screen a collection of 24 yeast and 23 bacterial strains isolated from kefir. The Toll-like receptor 5 agonist, flagellin was used to activate the reporter cells, while pre-incubation with the selected strains was tested to identify strains with the capacity to inhibit cell activation. In this system, 21 yeast strains from the genera *Saccharomyces*, *Kluyveromyces* and *Issatchenkia* inhibited almost 100% of the flagellin-dependent activation, whereas only some lactobacilli strains showed a partial effect. *K. marxianus* CIDCA 8154 was selected for further characterization. Inhibitory activity was confirmed at transcriptional level on Caco-2/TC-7 and HT-29 cells upon flagellin stimulation. A similar effect was observed using other pro-inflammatory stimulation such as IL-1β and TNF-α. Pre-incubation with yeasts induced a down-regulation of NF-κB signalling in epithelial cells in vitro, as well as expression of other pro-inflammatory chemokines such as CXCL8 and CXCL2. Furthermore, modulation of CCL20 mRNA expression upon flagellin stimulation was evidenced *in vivo*, in a mouse ligated intestinal loop model.

Results indicate kefir contains microorganisms able to abolish the intestinal epithelial inflammatory response that could explain some of the properties attributed to this fermented milk.
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1. Introduction

Intestinal epithelium is constantly exposed to a vast community of microorganisms that colonizes mammalian gastrointestinal tract and is a key player in the maintenance of gut homeostasis (McCole and Barrett, 2007). Due to its strategic location, it contributes of significantly to mucosal innate defence mechanisms. Upon recognition of specific microbial components and in a context of microbial invasion, it triggers a gene expression program that is aimed at orchestrating the immune response to block the microbial menace (Sansonetti, 2004).

In recent years, a myriad of products have been released for public consumption containing live microorganisms claimed to have probiotic activity. The scientific and clinical interest to find microorganisms with ability to regulate intestinal immune response has increased because of the accumulating evidence that the luminal flora plays a critical role in the initiation and perpetuation of inflammatory bowel diseases (IBD) (Strober et al., 2007). Although diverse beneficial effects are attributed to different microorganisms the mechanisms by which they exert their actions are not totally understood (Boirivant and Strober, 2007). Different studies have evidenced the capacity of bacterial species (mostly *Lactobacillus* and *Bifidobacterium* strains) to modulate the production of cytokines and pro-or anti-inflammatory mediators by dendritic cells (Foligne et al., 2007), macrophages (Marcinkiewicz et al., 2007) and intestinal epithelial cells (O'Hara et al., 2006; Toki et al., 2008). Although yeasts have been used traditionally for producing different foods and are present as commensals in the gastrointestinal tract, only *Saccharomyces boulardii* has been shown to have immune modulating

* Corresponding author.
E-mail addresses: dromanin@biol.unlp.edu.ar (D. Romanin), maserr@biol.unlp.edu.ar (M. Serradell), dolores@biol.unlp.edu.ar (D. González Maciel), nlausada@atlas.med.unlp.edu.ar (N. Lausada), ggarrote@biol.unlp.edu.ar (G.L. Garrote), martinr@biol.unlp.edu.ar (M. Rumbo).

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properties. *S. boulardii* is a non-pathogenic yeast used for many years to prevent or treat a variety of human gastrointestinal disorders (Buts, 2008; Buts and De Keyser, 2006; Szajewska et al., 2007; Zanello et al., 2008).

Kefir is obtained by milk fermentation with a mixed microflora confined to a matrix of discrete "kefir grains". Lactic acid bacteria (lactobacilli, lactococci, leuconostoc), acetic acid bacteria and several genera of yeast are present in this mixed microflora (Angulo et al., 1993; Garrote et al., 2001). Kefir consumption has been associated to several health-promoting properties such as antimicrobial, antitumoral, immunological and hypocholesterolemic effects and is empirically used in many eastern European regions to treat different gastrointestinal disorders (Farnworth, 2005).

Our workgroup has isolated and characterized more than 80 bacterial and yeast strains from kefir grains. Lactic acid bacteria strains showed differences in surface and probiotic properties such as resistence to bile salts and low pH, adhesion to Caco-2 cells and inhibitory power against intestinal pathogens in in vitro assays (Golowczyc et al., 2007, 2008). Several yeast strains isolated from kefir share these properties (G. Garrote, unpublished results).

In view of its microbial complexity and putative benefits derived from its consumption, kefir becomes a suitable source of potentially probiotic microorganisms. The current study was designed to select microorganisms isolated from kefir with capacity to down-regulate innate response on intestinal epithelial cells. We have used a reporter cell line developed by our group that induces the expression of firefly luciferase upon activation of innate response (Nempont et al., 2008). Using this system we could detect several yeast strains isolated from kefir that show high capacity to modulate epithelial innate response and we have further characterized this activity in different in vitro and *in vivo* systems.

2. Material and methods

2.1. Epithelial cell lines and reagents

Human colonic epithelial (Caco-2 and HT-29) cell lines were a kind gift from Dr. J.P. Kraehenbuhl. Caco-2 cells stably transfected with a luciferase reporter construction under the control of CCL20 promoter (Caco-2 ccl20: luc) were previously described (Nempont et al., 2008). Growing conditions of cell lines and flagellin (FliC) purification from *Salmonella enterica* were previously described (Anderle et al., 2005; Nempont et al., 2008), (Sierro et al., 2001). Human IL-1β and TNF-α were purchased from R&D (USA) and purified *Escherichia coli* LPS from Sigma Chemicals (USA).

2.2. Microbial culture

Yeast strains were cultured in YM agar (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l glucose, 20 g/l agar) at 30 ° C for 24 h. *Lactobacillus* strains were cultured in MRS broth (Biokar Diagnostics, Beauvais, France) at 30 °C for 48 h.

Lactobacillus plantarum DSMZ 20174, *L. casei* DSMZ 20011, *L. hilgardii* DSMZ 20176 and *L. acidophilus* DSMZ 20079 were from the German Collection of Microorganisms and Cell Culture; *L. acidophilus* ATCC314 and *L. brevis* ATCC 8287 from American Type Culture Collection; *L. brevis* JCM 1059 from the Japanese Collection of Microorganisms and *S. boulardii* was isolated from a commercial probiotic preparation (Floratil, Merck, Germany).

Microorganisms were harvested in sterile phosphate buffered saline (PBS) and suspensions at $OD_{590} = 1.0$ were prepared in Dulbecco-modified Eagle Medium (DMEM), the base medium of Caco-2 cell culture (Nempont et al., 2008).

In some cases, bacterial and yeast cells were heat-killed (at 100 °C for 5 min) or UV-irradiated 17600 μws/cm^2 for 50 min) before incubation with epithelial cells.

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

Confluent Caco-2 ccl20:luc cells were co-cultured 2 h with a suspension ($OD_{590} = 1$) of the microorganism to be tested. This OD corresponds roughly to 10^8 yeast/ml or 10^9 bacteria/ml as determined by plating for the different microorganisms studied. Cells were stimulated with FliC (1 μg/ml) for 6 h. Luciferase activity was measured in a Labsystems Luminoskan TL Plus luminometer (Thermo Scientific, USA) using a luciferase assay system (Promega, Madison WI, USA) as described (Nempont et al., 2008). Luminescence was expressed as percentage of the mean of stimulated control (NAL).

2.4. In vitro analysis of chemokine expression

Cell monolayers were incubated for 1 h with *Kluyveromyces marxianus* CIDCA 8154 strain at $OD_{590} = 1.0$ as described above. Then cells were stimulated with FliC (1 μg/ml), IL-1β (10 ng/ml), TNF-α (100 ng/ml) or LPS (1 ng/ml). Two hours after stimulation, samples were homogenized in RA1 lysis buffer (GE Healthcare, Germany) to perform the extraction of total RNA as described below.

2.5. Transient transfection of the Caco-2 cells

Caco-2 cells were transfected using Lipofectamine™ 2000 (Invitrogen, USA) with plasmids containing *Renilla* spp. luciferase under control of HSTK promoter and firefly luciferase under a NF-κB dependent promoter (3X-κB artificial promoter) (Felley-Bosco et al., 2000). Transfected cells were pre-incubated with yeast and then stimulated with FliC as described before. After treatments, luminescence was measured using Dual Luciferase Assay kit (Promega, USA).

2.6. Mouse ligated intestinal loop model

Seven week-old BALB/c female mice (4 animals per group), were used. Animals were housed in a climate-controlled room on a 12-h light–dark cycle, with food (standard laboratory mice chow) and water *ad libitum*. All animal experiments were performed according to the guidelines set by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Mice were anesthetized with intraperitoneal administration of diazepam (5 mg/kg), and ketamine (100 mg/kg). A 7 cm section of small intestine from duodenum was clamped and 50 μl of PBS or *K. marxianus* CIDCA 8154 suspension ($4.6 \pm 0.9 \times 10^8$ cfu/ml) was injected into the lumen. Thirty minutes later, 150 μl of FliC (450 μg) or PBS was injected into the same intestinal ligated loop section, and the abdominal wall was sutured. During the procedure, mice were kept warm on a 37 °C warming pad. Ninety minutes after the second injection, mice were sacrificed by cervical dislocation and 1-cm section of the intestinal ligated loop was excised. Upon washing with ice chilled PBS and removal of Peyer's patches, intestinal sections were homogenized in a RA1 lysis buffer (GE Healthcare, Germany) to perform the extraction of total RNA. The samples were analyzed by real-time PCR for CCL20 mRNA as described below.

2.7. Real-time PCR analysis

Total RNA extraction was performed using the NucleoSpin RNA II kit (Macherey-Nagel, Germany). Reverse transcription was performed using random primers and MMLV-Reverse transcriptase (Invitrogen, USA). Real-time PCR was performed following manufacturer's protocol using the iCycler thermal cycler (BioRad, USA). Primers for CCL20 (Mip3α , interleukin-8 (IL-8), CXCL2 (Mip-2α), lactase phloryzin hidrolase, fractalkine (CX3CL1) and mouse actin or human histone 3.3 and relative difference calculation using the ΔCt method were previously described (Rumbo, et al, 2004; Anderle et al., 2005). For detection of intestinal trefoil factor (ITF), Cdx2 and macrophage

inhibitory factor (MIF) we used the following primers: ITFfwd: TCCTGGCCTTGCTGTCCTC, ITFrev: ACGGCACACTGGTTTGCAG; CDX2fwd: AGAAGTGTCCCAGAGCCCTTG, CDX2rev: CAGGGACAGAGCCAGACACTG; MIFfwd: GTTCCTCTCCGAGCTCACCCAGCAGC, MIFrev: GCAGCTTGCTGTAGGAGCGGTTCT.

2.8. Statistical analysis

Differences in luciferase activity and mRNA expression level were statistically tested by using one way ANOVA and Student *t*-test to determine any significant difference.

3. Results

3.1. Down-regulation of Caco-2 ccl20 reporter by microbial strains isolated from kefir

The ccl20 promoter is highly inducible in Caco-2 cells upon different pro-inflammatory stimuli such as TLR agonists, IL-1β or TNF-α (Anderle et al., 2005), being a sensitive indicator of activation of innate response (Nempont et al., 2008). Ccl20 promoter activity is dependent of NF-kB, AP1 and ESE-1 binding sites present in the −300 to −100 bp region (Kwon et al., 2003). A collection of 23 bacterial strains and 24 yeast strains isolated from kefir were screened using a Caco-2 ccl20:luc reporter system. A very strong inhibition of luciferase activity was observed by a 2 h pre-incubation with 21 of the assayed yeast (Table 1 marked *, $p<10^{-8}$, less than 10% of activity of the cells stimulated in absence of microorganism), whereas the 3 remaining strains produced strong inhibition ($p<10^{-5}$, residual activity of 10% to 40% of activity obtained without microorganism pre-incubation). On the other hand, only 8 bacterial strains were able to induce a 45–75% of inhibition of luciferase activity (Table 1, marked †) and slight inhibitory capacity was evidenced in another 10 bacterial strains (Table 1 marked *). Yeasts isolated from kefir belonging to different genera, as well as *S. boulardii* isolated from a commercial probiotic formula included here as control, were capable to reduce the cell activation induced by FliC to almost basal level while none of the bacteria assayed showed this inhibitory power.

3.2. Inhibitory effect on response to pro-inflammatory stimuli in Caco-2 and HT-29 cells

Since yeast strains seem to have a great capacity to down-modulate innate activation, we selected strains able to resist gastrointestinal tract conditions for further characterization. Among them, *K. marxianus* CIDCA 8154 showed the best resistance to 1% bile salts and hydrochloride acid and the capacity to survive along the gastrointestinal tract passage upon oral feeding (unpublished data). Pre-incubation of Caco-2 cell monolayer with this yeast produced a (30)-fold inhibition of the FliC-induced CCL20 mRNA expression, almost abrogating completely the induction of CCL20 expression due to FliC stimulation (Fig. 1a). Similar levels of inhibition were observed when other pro-inflammatory stimuli such as IL-1β and TNF-α were used (Fig. 1a). Expression levels of other genes normally expressed by enterocytes were not affected by either flagellin stimulation or yeast pre-treatment (Fig. 1b). Expression of genes related with cellular differentiation (Cdx2), enterocyte digestive function (lactase phlorizin hydrolase, LPH) (Mitchelmore et al., 2000), epithelial repair (intestinal trefoil factor, ITF) (Taupin et al., 2000) or constitutive chemokines (MIF) (Maaser et al., 2002) were not affected by yeast pre-incubation, indicating a specific effect of yeasts on the inhibition of pro-inflammatory pathways. In order to test activity on another intestinal epithelial cell line, HT-29 cells were used. A significant decrease of CCL20 mRNA induction was observed when HT-29 cells were incubated with *K. marxianus* CIDCA 8154 prior to activation with LPS, IL-1β or FliC (Fig. 1c). Pre-incubation with the yeasts was also able to inhibit the mRNA expression of chemokines CXCL8 (IL-8) and CXCL2 (MIP2-α) upon stimulation with either FliC or IL-1β in Caco-2 cells (Fig. 1d), whereas a similar trend was observed for CX3CL1 expression, with lower statistical significance. Overall, these results indicate a general anti-inflammatory effect over epithelium induced by pre-incubation with yeasts.

Table 1
Modulation of pro-inflammatory response in Caco-2 ccl20:luc reporter system by yeast and bacterial strains isolated from kefir.

Controls				Lactic acid bacteria			
	NAL		SD		NAL		SD
Basal Activity	1.28	±	0.00	Lp 8324†	48.07	±	0.21
FliC stimulated	100	±	0.17	Lk 8346†	50.12	±	0.26
				Lp 8337†	54.13	±	0.14
Yeasts				Lk 8316†	61.61	±	0.04
	NAL		SD	Lk 8314†	66.28	±	0.23
Sc 81109*	0.72	±	0.00	Lp 83112†	67.52	±	0.01
Km 81111*	1.68	±	0.01	Lp 8312†	71.46	±	0.27
Km 8116*	2.00	±	0.00	Lp 83114†	74.52	±	0.19
Km 8118*	2.39	±	0.01	Lk 83115††	76.26	±	0.18
Km 81105*	2.40	±	0.00	Lk 83111††	77.65	±	0.15
Km 8153*	2.58	±	0.01	Lp 83210††	79.27	±	0.20
Km 8154*	2.72	±	0.01	Lp 8338††	80.74	±	0.20
Sc 81106*	2.74	±	0.01	Lk 8319††	83.28	±	0.17
Km 8113*	3.01	±	0.02	Lk 8317††	86.33	±	0.20
Sc 8112*	3.04	±	0.00	Lk 83110††	90.01	±	0.41
Sc 9127*	3.49	±	0.01	Lk 8315††	90.89	±	0.14
Km 81104*	3.64	±	0.02	Lk 83116††	93.08	±	0.22
Km 9121*	4.63	±	0.01	Lk 8310††	93.33	±	0.14
Sc 9123*	4.64	±	0.01	Lp 8323	98.95	±	0.25
Sc 9136*	4.88	±	0.02	Lk 8348	99.85	±	0.17
Sc 9133*	5.24	±	0.01	Lk 8321	104.67	±	0.21
Sc 9124*	5.83	±	0.02	Lk 8344	110.85	±	0.08
Is 9131*	6.46	±	0.00	Lk 83113	117.62	±	0.15
Sc 81103*	6.59	±	0.03	Collection strains			
Sc 9132*	8.23	±	0.03	Lb JCM 1059†	45.86	±	0.05
Sc 81108*	9.40	±	0.00	Lc DSMZ 20011†	71.27	±	0.06
Sc 81102**	12.70	±	0.16	Lp DSMZ 20174††	77.79	±	0.26
Sc 8175**	21.39	±	0.15	La ATCC 8287††	85.54	±	0.04
Sc 8111**	33.98	±	0.21	La DSMZ 20079††	90.97	±	0.03
Collection strains				La ATCC314	108.97	±	0.07
*S. boulardii**	7.63	±	0.02	Lh DSMZ 20176	121.82	±	0.01

NAL: normalized average luminescence expressed as percentage of activity induced with flagellin stimulation. Caco-2/Tc-7 ccl20:luc reporter cells were pre-incubated with different microorganisms and stimulated with flagellin. Results are expressed as mean ± SD and are representative of at least three independent experiments.
Sc *Saccharomyces cerevisiae*; Km *Kluyveromyces marxianus*; Is *Issatchenkia* spp; Sb *Saccharomyces boulardii*; La *Lactobacillus acidophilus*; Lb *Lactobacillus brevis*; Lc *Lactobacillus casei*; Lh *Lactobacillus hilgardii*; Lk *Lactobacillus kefir*; Lp *Lactobacillus plantarum*.
* Significant inhibition ($p<10^{-8}$), residual activity less than 10% of activity obtained without microorganism pre-incubation.
** Significant inhibition ($p<10^{-5}$), residual activity of 10% to 40% of activity obtained without microorganism pre-incubation.
† Significant inhibition ($p<0.001$), residual activity of 45% to 75% of activity obtained without microorganism pre-incubation.
†† Significant inhibition ($p<0.05$), residual activity of 75% to 95% of activity obtained without microorganism pre-incubation.

3.3. Effect of viability, doses and pre-incubation time

A dose–response of inhibitory activity upon incubation with either *S. cerevisiae* CIDCA 8112 or *K. marxianus* CIDCA 8154 was observed (Fig. 2a). Additionally, viability of the different yeasts analyzed was essential to keep this activity since the modulatory effect on Caco-2 ccl20:luc cells was completely abolished by heat-inactivation or UV-irradiation when an experimental setting similar to the one employed in the screening was used (Fig. 2b).

A time-course experiment using different pre-incubation time of epithelial cells with yeast strains was performed. We observed that both microorganisms keep their inhibitory capacity even when they

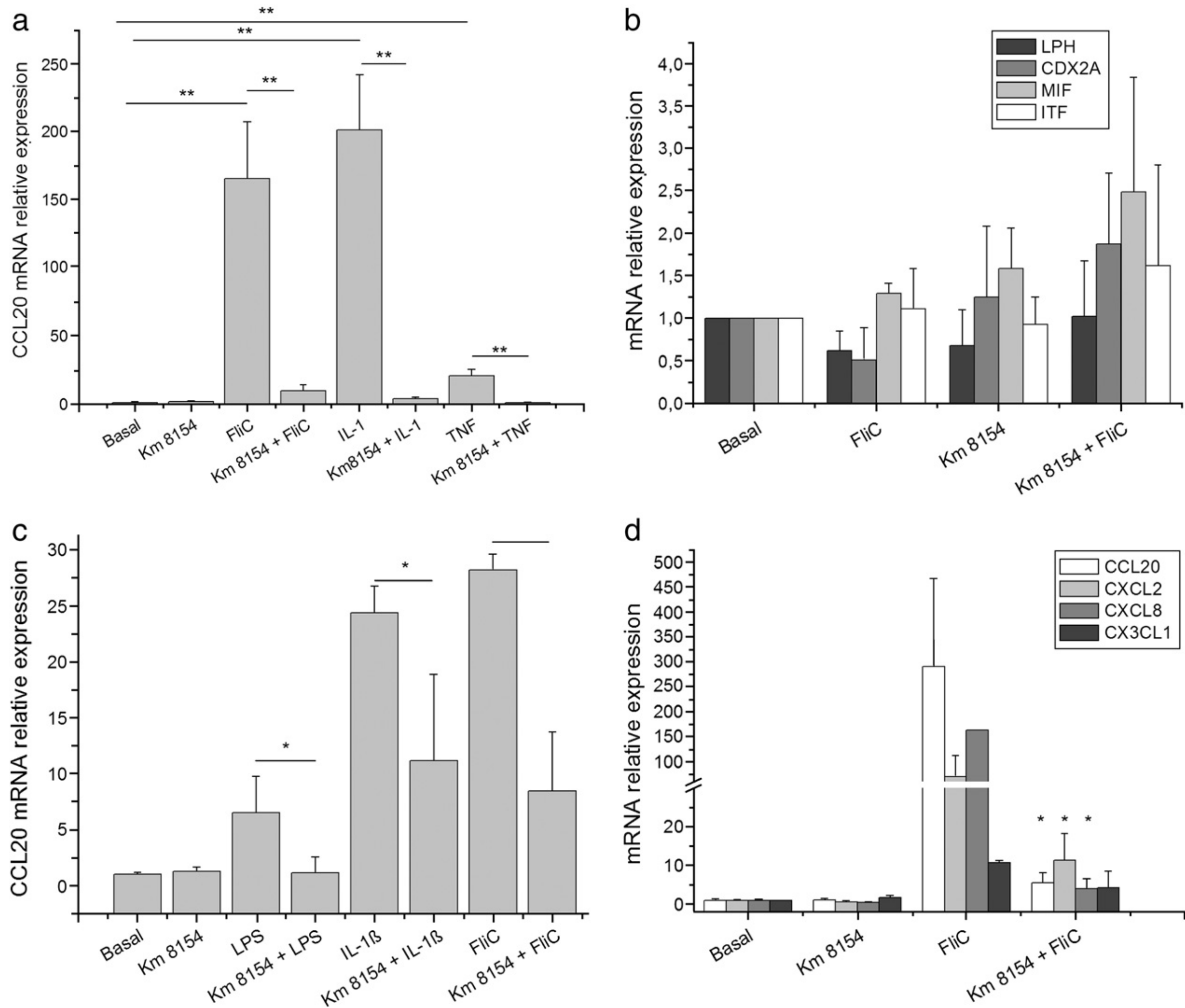

Fig. 1. Effect of *K. marxianus* CIDCA 8154 (Km 8154) on: a. Expression of ccl20 mRNA on Caco-2/Tc-7 cells (**$p<0.01$). b. Gene expression levels related to different cell functions on Caco-2/Tc-7 cells. c. Expression of ccl20 mRNA on HT29 cells. d. Chemokine mRNA on Caco-2/Tc-7 cells. Cells were incubated with Km 8154 and stimulated with flagellin (FliC), IL-1β or *E. coli* purified LPS. Results are expressed as mean ± SD and are representative of at least two independent experiments. (*$p<0.05$). β-actin was used to normalize gene expression.

were added at the same time that the stimulus, without pre-incubation time (Fig. 2c). Furthermore, even when they are added 1 h after stimulation the modulatory effect on innate activation is evident.

Since activity of the CCL20 promoter may rely on different signalling pathway activations (Kwon et al., 2003), we used a transient transfection with a reporter driven by an artificial promoter including 3-κB binding sites in tandem controlling luciferase activity in order to analyze the effects exclusively on NF-κB pathway activity (Felley-Bosco et al., 2000). In this system, we confirmed that pre-incubation with *K. marxianus* CIDCA 8154 is able to abolish flagellin-induced NF-κB activation (Fig. 2d).

3.4. Effects on response to flagellin in polarized cultures

On polarized Caco-2 ccl20:luc cultures, K marxia*nus* CIDCA 8154 was able to down-regulate epithelial cell activation by basolateral FliC stimulation when yeasts was incubated either on apical or basolateral side of monolayer (Fig. 3). This indicates that the down-modulatory effect is induced by yeast-epithelial cell interaction and not merely caused by sequestration of the inflammatory stimulus by the yeasts. Since these results could be explained by a cell-to-cell contact mechanism or by a soluble factor released by yeasts as described for *S. boulardii* (Sougioultzis et al., 2006), we performed some additional experiments using yeast-conditioned cell culture medium. Any of the conditions tested were able to produce a significative inhibition of luciferase activity in Caco-2 ccl20:luc cells (data not shown).

3.5. Effects on response to flagellin in in vivo models

To evaluate the ability of *K. marxianus* CIDCA 8154 to down-regulate the FliC-induced CCL20 mRNA expression *in vivo*, we performed a mouse ligated intestinal loop experiment. FliC-treated mice showed almost a 3-fold increase in the expression of CCL20 mRNA in the loop, while no stimulation was observed in PBS-injected or yeast-injected control groups (Fig. 4). However, the intraluminal administration of yeasts 30 min before stimulation reduced the CCL20 mRNA expression to levels comparable with the non stimulated control.

4. Discussion

We investigated the ability of different potentially probiotic yeasts and bacteria isolated from kefir to modulate the response to pro-inflammatory stimuli on intestinal epithelial cells. In our screening system, all the yeast strains analyzed and some strains of *Lactobacillus*

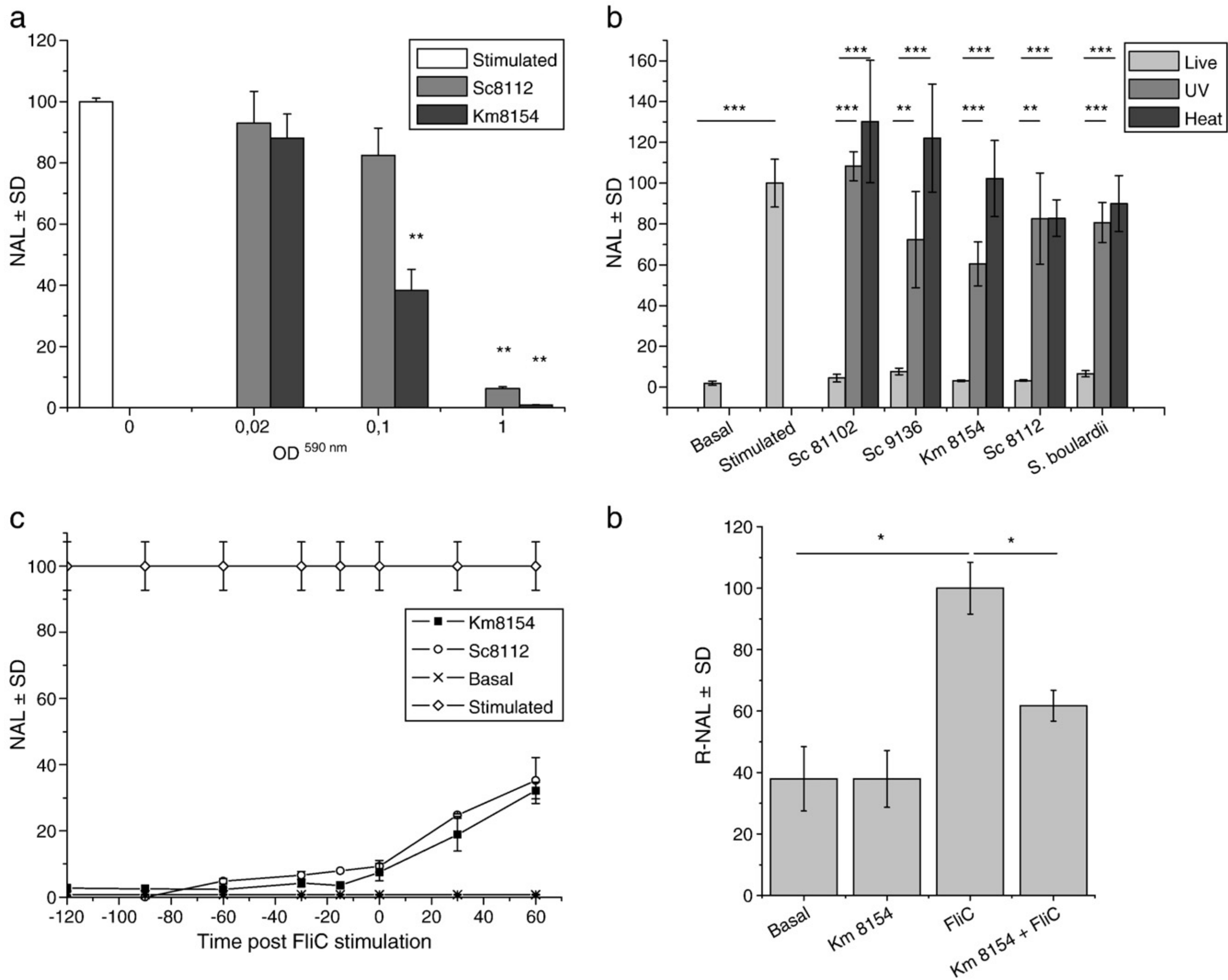

Fig. 2. Effect of doses, viability and pre-incubation time of microorganisms over ccl20 expression. a. Effect of the dose of *K. marxianus* CIDCA 8154 (Km 8154) or *Saccharomyces cerevisiae* CIDCA 81102 (Sc 81102) on the Caco2 ccl20:luc reporter system stimulated with flagellin. b. Effect of live (light gray), UV (gray) and heat-inactivated (dark gray) microorganism on the Caco-2 ccl20:luc reporter system. c. Effect of pre-incubation time with kefir microorganisms on the Caco-2 ccl20:luc. Data are representative of three independent experiments. d. Effect of incubation with Km 8154 on Caco-2/Tc-7 cells transiently transfected with 3x-κB:luc and HSV-TK:renilla luciferase reporters. *Renilla* luciferase activity was used to normalize the transfection efficiency. Data are expressed as a percentage of the activity observed when flagellin alone was used as treatment (R-NAL), as indicated in Table 1. Data is expressed as mean ± SD of two independent experiments. (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

were able to inhibit the flagellin-induced activation. Furthermore, lactic acid bacteria were far less effective in preventing the activation when compared to yeast strains, considering that we used a microorganism:cell ratio higher in the former case (10^8 bacteria cfu/ml vs 4.0×10^7 yeast cfu/ml) and percentage of inhibition caused by yeasts was always much higher. Among the 18 bacterial strains that showed any down-modulatory capacity, 11 were of *Lactobacillus kefir*, being the first report on the modulatory capacity of this species so far. On the other hand, all the kefir-derived yeast strains showed the capacity to inhibit almost completely the reporter activation, strongly depending on the viability of the microorganism. Yeasts from the genera *Saccharomyces*, *Kluyveromyces* and *Issatchenkia* greatly inhibited epithelial cell innate activation. Furthermore, the non-kefir-derived yeast *S. boulardii*, included as control based on its described anti-inflammatory properties (Sougioultzis et al., 2006), showed comparable activity. Our results indicate that inhibition of innate epithelial response could be a rather general property of different yeast species, particularly represented among kefir microflora.

Based on its resistance to gastrointestinal tract conditions, we selected a *K. marxianus* strain to characterize its anti-inflammatory capacity. It was able to inhibit the expression of several chemokines triggered by different TLR ligands such as flagellin and LPS and abolished the expression of the same set of markers induced by TNF-α and IL-1β. On the other hand, yeast pre-incubation did not affect expression of several enterocyte-specific genes. Similar activity was observed in other cell lines and in primary mouse enterocytes culture (data not shown), indicating a wide capacity to down-modulate the pro-inflammatory epithelial response. Furthermore, the results observed cannot be attributed to a toxic effect of yeast or yeast products on the epithelial cells since neither the housekeeping gene expression levels, nor the other different genes tested to assess different enterocytic function altered their expression levels upon pre-incubation with the yeasts.

Using a specific NF-κB reporter driven by an artificial promoter, we showed that *K. marxianus* is able to block the activation of this pathway, suggesting that its broad modulatory activity may be mediated by this mechanism, being the first report of this capacity in yeasts from the *Kluyveromyces* genus. Sougioultzis et al. (2006) demonstrated that *S. boulardii* produces a small molecular weight, water soluble factor that inhibits NF-κB-mediated IL-8 gene expression *in vitro*. In our system we could not evidence the presence of soluble factors mediating this effect, although we cannot formally exclude this possibility. Our results show that NF-κB modulation is not an exclusive property of *S. boulardii* and may be shared by other yeast

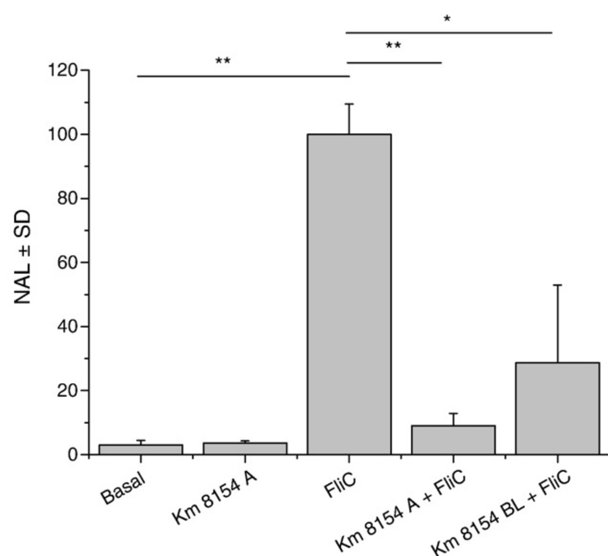

Fig. 3. Effect of incubation with *Kluyveromyces marxianus* CIDCA 8154 (Km 8154) in polarized Caco-2 ccl20:luc cultures. A: apical side. BL: basolateral side. Data are representative of three independent experiments. (*$p<0.05$, **$p<0.01$).

species. Further studies on the signalling mechanisms mediating these effects are awaited.

We confirmed that the modulatory activity can act from the apical side of a polarized culture, indicating that this could also operate from the intestinal lumen. Furthermore, using a ligated ileal loop model and flagellin intraluminal administration, could we confirm the *in vivo* effects of *K. marxianus* CIDCA 8154. Only after delivering high amounts of intraluminal flagellin in the ligated ileal loop model, we could detect an activation of CCL20 expression in the intestine, possibly due to the low responsiveness to flagellin when administered on the luminal side, as is well documented in epithelial cell lines (Gewirtz et al., 2001; Zeng et al., 2003). Under these settings, the presence of *K. marxianus* in the intestinal lumen abolishes flagellin-induced CCL20 activity, indicating that the yeast down-regulatory capacity can operate in the intestinal tract conditions.

Taking into account the different microbial populations present in kefir, it is pretty complicated to anticipate if the reported activity of yeasts isolated from kefir could be reflected upon consumption of the fermented milk. Although kefir is believed to have several health-promoting properties, there are few reports on the effects of kefir administration, most of them being performed on naive mice models.

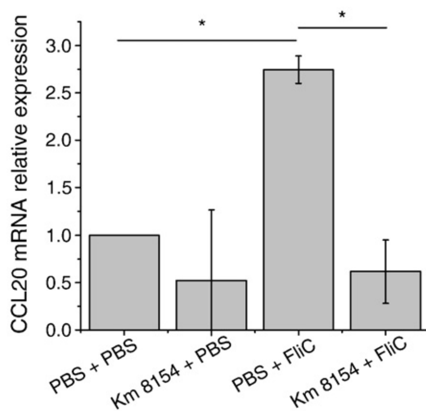

Fig. 4. *Kluyveromyces marxianus* CIDCA 8154 (Km 8154) activity *in vivo* on a mouse ligates intestinal loop model stimulated with flagellin. Data is expressed as mean ± SE of three independent experiments ($p<0.05$). β-actin was used to normalize gene expression.

The immunostimulating capacity of kefir was demonstrated in different animal models (Thoreux and Schmucker, 2001; Vinderola et al., 2005; Vinderola et al., 2006). In particular, Vinderola et al. (2006) described the increase of IgA^+, $IL\text{-}4^+$, $IL\text{-}6^+$ and $IL\text{-}10^+$ cell numbers in intestinal mucosa and an improved phagocytic capacity of peritoneal macrophages in BALB/c mice upon kefir administration. Studies have been performed with complete kefir or kefir supernatant but not with isolated microorganisms. These studies were not specifically designed to demonstrate an innate response modulatory capacity as reported here.

Several studies have shown that certain probiotics may be useful in the treatment and prevention of a number of intestinal disorders including IBDs. Successful treatment has been reported with *E. coli* Nissle 1917 in patients with ulcerative colitis (UC) (Schultz, 2008) and a multiple probiotic organism's mixture (VSL#3) has been useful for management of Crohn's disease, UC and pouchitis (Chapman et al., 2006). Furthermore, the administration of the probiotic yeast *S. boulardii* in drinking water has been effective to diminish the inflammation in an acute model of colitis caused by intrarectal treatment with TNBS (Lee et al., 2008). All these evidence indicates that in certain cases, the administration of live microorganisms may be an alternative to treat specific gastrointestinal disorders.

We have shown here that yeasts isolated from kefir showed a high capacity to inhibit intestinal epithelial innate response triggered by different pro-inflammatory pathways, whereas this activity was only moderate in different lactic acid bacteria analyzed. This activity seems to be dependent on NF-κB modulation and was evidenced either *in vitro* or *in vivo*. Kefir-derived yeasts could provide an alternative for management of inflammatory gastrointestinal disorders, upon validation in adequate experimental models and randomized trials.

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