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# Probiotic yeast *Kluyveromyces marxianus* CIDCA 8154 shows anti-inflammatory and anti-oxidative stress properties in *in vivo* models

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**RESEARCH ARTICLE**

## Abstract

Inflammatory bowel diseases (IBDs) are complex affections with increasing incidence worldwide. Multiple factors are involved in the development and maintenance of the symptoms including enhanced oxidative stress in intestinal mucosa. The conventional therapeutic approaches for IBDs are based on the use anti-inflammatory drugs with important collateral effects and partial efficacy. In the present work we tested the anti-inflammatory capacity of *Kluyveromyces marxianus* CIDCA 8154 in different models. *In vitro,* we showed that the pretreatment of epithelial cells with the yeast reduce the levels of intracellular reactive oxygen species. Furthermore, in a murine model of trinitro benzene sulfonic acid-induced colitis, yeast-treated animals showed a reduced histopathological score ($P<0.05$) and lower levels of circulating interleukin 6 ($P<0.05$). The capacity to modulate oxidative stress *in vivo* was assessed using a *Caenorhabditis elegans* model. The yeast was able to protect the nematodes from oxidative stress by modulating the SKN-1 transcription factor trough the DAF-2 pathway. These results indicate that *K. marxianus* CIDCA 8154 could control the intestinal inflammation and cellular oxidative stress. Deciphering the mechanisms of action of different probiotics might be useful for the rational formulation of polymicrobial products containing microorganisms targeting different anti-inflammatory pathways.

**Keywords:** innate immunity, probiotic yeast, inflammatory bowel disease.

## 1. Introduction

Inflammatory bowel diseases (IBDs), mainly represented by ulcerative colitis (UC) and Crohn's disease (CD), are chronic debilitating conditions that are difficult to treat. In the last few decades, they have an increased global incidence, especially in developing countries (Molodecky *et al.*, 2012). IBDs are complex and multifactorial affections; it was demonstrated that diet, age, genetic background, ethnicity, and clinical history of infections among others influence in the incidence and morbidity of the disease (Cabré and Domènech, 2012; Kaser *et al.*, 2010). It is a widely held view that in IBDs and in CD in particular, the mucosal immune system inappropriately reacts against the normal commensal microbiota (Alfa *et al.*, 2002) and some associations have been reported, for example it was shown that Crohn's disease patients are frequently colonised by adherent-invasive *Escherichia coli* (Chassaing and Darfeuillemichaud, 2011). Some symbiotic bacterial species have been shown to prevent intestinal inflammatory host responses, but the physiologic intestinal microbiota also contains microorganisms that have been shown to induce inflammation under particular conditions (Antonopoulos *et al.*, 2009; Asquith and Powrie, 2010; Atarashi *et al.*, 2011). It has been shown that IBDs are linked with oxidative stress in the gut epithelia, which may be involved in the development of ulcerative colitis-associated carcinogenesis. This process is contributed by DNA damage induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Lih-Brody *et al.*, 1996; Roessner *et al.*, 2008; Seril, 2003) that are generated in the intestinal mucosa and released as proinflammatory mediators. These species can damage the intestinal barrier and expose the mucosal immune system to luminal content, which enhances the inflammation

(Almenier *et al.*, 2012; Grisham, 1990; Hollander, 1992; Simmonds *et al.*, 1992). The oxidative damage in IBD patient samples has been manifested by detecting the presence of products from lipid peroxidation reactions (Almenier *et al.*, 2012; Chiarpotto *et al.*, 1997; Rumley and Paterson, 1998; Sedghi *et al.*, 1994).

The conventional approach to managing active IBD has been based on progressive intensification of therapy with different anti-inflammatory drugs as disease worsens (Dignass *et al.*, 2010, 2012). Resection surgery of affected segments is an alternative that can be considered. Patient management is focused on inducing and maintaining clinical remission, allowing withdrawal of corticosteroids and preventing post-operative recurrence of disease. Several recombinant cytokines and other biologicals are under clinical trials to treat some aspects of the disease. One main concern related to these options is the increasing cost that health systems have to face in the use of sophisticated therapeutic approaches; any of them has been shown to be curative so far. In parallel, some proposed therapeutic strategies to complement the treatments previously mentioned come from the field of nutrition. Diet supplements including ω-3 fatty acids, the plant-derivate terpen celasterol and probiotic microorganisms have been reported to diminish the oxidative stress, down-modulate inflammation and protect from damage in different models of IBDs (Barbosa *et al.*, 2003; Genove *et al.*, 2012; Shaker *et al.*, 2014). Although several clinical studies have been conducted using different probiotic microorganisms in specific situations, there is still no consensus in the type of probiotic to be used and under which circumstances. Among the different IBDs, UC seems to benefit from the use of probiotics as complementary therapy, although there is still much place for further analysis (Scaldaferri *et al.*, 2013). There are several mechanisms by which probiotic exert their beneficial role that have been only partially characterised and presumably are different depending on the microbial species considered (Bermudez-Brito *et al.*, 2012; Vieira *et al.*, 2013). Such complexity has circumscribed the application of probiotics to empiric approaches to complement conventional therapy in specific pathologies (Sanders *et al.*, 2013). The use of animal models aimed to characterise aspects of host-probiotic interaction is a valuable tool to select probiotic strains for product development and predict their effects when applied to specific physiopathological situations (Nanau and Neuman, 2012).

We have previously shown that different yeast strains isolated from kefir fermented milk show high capacity to modulate intestinal epithelial inflammatory activation *in vitro.* We selected *K. marxianus* CIDCA 8154 since it showed the highest anti-inflammatory capacity (Romanin *et al.*, 2010), while presenting other probiotic qualities, such as bile acid resistance, capacity to survive the passage along the gastrointestinal tract and good growing profile in different culture media and conditions (Diosma *et al.*, 2014). In the present work, we further characterise the activity of this strain using animal models useful to predict its capacity to modulate specific intestinal inflammatory pathology and gain insight in its mode of action.

## 2. Material and methods

### Microorganisms

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.

### *Caenorhabditis elegans* strains and maintenance conditions

*Caenorhabditis elegans* strains N2, Bristol (wild-type); CB1370, daf-2 (e1370); GR1307, daf-16 (mgDf50); and LG333, skn-1, (zu135) were obtained from the Caenorhabditis Genetics Center at the University of Minnesota and maintained at 20 °C on nematode growth medium (NGM; USBiological, Swampscott, MA, USA). Strain *E. coli* OP50 used as normal diet for nematodes was requested from the Caenorhabditis Genetics Center.

### Cell culture and ccl20:luciferase reporter assay

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 interleukin (IL)-1β and tumour necrosis factor alpha (TNF-α) were purchased from R&D (Minneapolis, MN, USA) and purified *Escherichia coli* LPS from Sigma Chemicals (St. Louis, MO, USA). Confluent Caco-2 ccl20:luc cells growth on 48-well plates were incubated with a optical density $(OD)_{590nm}$ 1 suspension of the microorganism, equivalent to $1\times10^6$ cfu/ml, for 30 min. After incubation the stimulus is added to the culture at a final concentration of 1 µg/ml for FliC, 10 ng/ml for TNF-α and 100 ng/ml for IL-1β. After 6 h cells were collected and luciferase activity was evaluated using the Luciferase Assay Kit (Promega, Madison, WI, USA) following manufacturer's instructions using a Luminoskan TL Plus luminometer. Normalised Average Luminescence (NAL) was calculated dividing each well measure by the mean of the stimulated control, thus the mean of the stimulated control represents 100% of stimulation.

### Detection of endogenous reactive oxygen species

Confluent Caco-2 cells were incubated 30 min with 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate acetyl ester (CM-$H_2$DCFDA) (Invitrogen, Waltham, MA, USA) 4 µM in phosphate buffered saline (PBS)-HEPES 10 mM at 37 °C 5% $CO_2$, 95% air, after two washes with fresh PBS epithelial cells were co-incubated 15 min with a suspension of OD 1 of the microorganism to be tested. Negative controls were incubated with PBS and positive controls using different concentrations of $H_2O_2$. An inverted fluorescence microscope was used to capture images (Nikon Eclipse; Nikon, Tokyo, Japan). For each individual experiment every condition was tested in three wells and at least three fields were randomly captured for each well. Image analysis was performed using ImageJ software (http://rsbweb.nih.gov/ij/) (Abràmoff *et al.*, 2004; Rasband, 1997), with an automated script. Fluorescence intensity was measured and referred to the area fraction occupied by epithelial cells. Fluorescence fold increase was calculated as the rate of fluorescence by area fraction of each condition referred to the mean of the basal levels, thus basal control takes a value of 1 and treatments may be read as fold-induction of fluorescence.

### Trinitrobenzene sulfonic acid induced colitis

Male 6-week Balb-c mice were offered *ad libitum* normal water (water pretreatment) or a *K. marxianus* CIDCA 8154 suspension containing $1\times10^6$ yeast/ml (yeast pretreatment) during three days, with daily changes of the suspension prior to a single intrarrectal administration of 2.5 mg of trinitrobenzene sulfonic acid (TNBS, Sigma) in 50% ethanol as vehicle with a final volume of 200 µl (TNBS challenge). Control animals were inoculated with 50% ethanol in distilled water (vehicle challenge) (Alex *et al.*, 2009a). Animals were sorted in four groups according to the administration of the pretreatment (PT) and the challenge (C) as follow water/vehicle, yeast/vehicle, water/TNBS and yeast/TNBS. Treatment with yeast was interrupted after challenge. After 48 h of TNBS inoculation, animals were sacrificed by cervical dislocation and samples of colon were taken for histological analysis (haematoxylin and eosin staining) and RNA extraction. Damage was estimated in a double blind manner according to the histopalogical activity index (HAI) described by Alex *et al.* (2009b). Briefly the epithelial damage was scored as 0 for none, 1 for a minimal loss of goblet cells, 2 for extensive loss of goblet cells, 3 for a minimal loss of crypts and extensive loss of goblet cells, and 4 points for extensive loss of crypts; the infiltration was scored as 0 for none, 1 for an infiltrate around crypt bases, 2 for an infiltrate in muscularis mucosa, 3 for extensive infiltrate in muscularis mucosa with oedema, and 4 points for the infiltration of submucosa. The index (HAI) was calculated as the sum of the epithelial damage and the infiltration score, ranging between 0 and 8 points from unaffected to severe colitis.

### Serum interleukin-6 determination

Before treatment, 24 h after TNBS treatment and at sacrifice, blood was collected by submandibular bleeding. Serum IL-6 determination was performed using BD Biosciences OptEIA™ Mouse IL-6 ELISA Kit (Franklin Lakes, NJ, USA), according to manufacturer instructions.

### RNA extraction, reverse transcription and real time qPCR

Total RNA extraction was performed using the NucleoSpin RNA II kit (GE Healthcare, Waukesha, WI, USA). Reverse transcription was performed using random primers and MMLV-Reverse transcriptase (Invitrogen). Real-time PCR was performed following manufacturer's protocol using the iCycler thermal cycler (BioRad, Hercules, CA, USA). Primers for CCL20 (Mip3α), interleukin-8 (IL-8), CXCL2 (Mip-2α), lactase phloryzin hidrolase, fractalkine (CX3CL1) and mouse actin or human actin and relative difference calculation using the ΔΔCt method were previously described (Anderle *et al.*, 2005; Rumbo *et al.*, 2004). 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

### Oxidative stress measurement in *Caenorhabditis elegans*

To measure *K. marxianus* CIDCA 8154 effect on oxidative stress, yeast cells were inoculated in fresh liquid YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose) and incubated overnight at 28 °C. Cells were harvested by centrifugation at 4,000 rpm for 5 min, and diluted to a final $OD_{600nm}$ of 12 in YPD broth. To measure *C. elegans* survival rates after exposure to oxidative stress, we employed synchronised eggs, hatched in NGM (nematode growth medium) plates containing the *E. coli* OP50 strain, in the absence or presence of *K. marxianus* CIDCA 8154 (30 µl of cell yeast suspension with $OD_{600nm}$ of 12). Nematode viability was assessed after oxidative stress (1.5 mM $H_2O_2$) following the methodology described by Martorell *et al.* (2013). Experiments were carried out in triplicate.

### Data analysis and statistics

Numerical data was analysed using GraphPad Prism (v 5.00; GraphPad Software, La Jolla, CA, USA). Statistical tests used are described in the figures legends.

## 3. Results

### Characterisation of the anti-inflammatory capacity of *Kluyveromyces marxianus* CIDCA 8154 *in vitro*

*K. marxianus* CIDCA 8154 was able to down-modulate the proinflammatory response by flagellin stimulation (Figure 1A) even after washing with PBS. In agreement to our previous results, viability of the microorganisms is an essential factor to control epithelial proinflammatory activation induced by IL-1β (Figure 1B, $P<0.005$) or flagellin (Romanin *et al.*, 2010), indicating an active role of the yeast in the immunomodulation. Commensal bacteria and certain *Lactobacillus* strains have been described to interact with intestinal epithelial cells inducing a ROS-mediated anti-inflammatory signalling (Kumar *et al.*, 2007; Lin *et al.*, 2009). To identify whether *K. marxianus* CIDCA 8154 is able to induce the generation of intracellular ROS in intestinal epithelial cells, Caco-2 cell lines were incubated with the redox-sensitive indicator CM-H2DCFDA and with the microorganism. The pre-incubation of the epithelial cells with the yeast caused a significant decrease of intracellular ROS ($P<0.005$). Preincubation with the yeast also diminished the fluorescence induced by exogenous $H_2O_2$, an effect that was dependent on microorganism viability (Figure 1 C and D). In contrast, *Lactobacillus plantarum* CIDCA 83114 from our collection, which showed moderate capacity to modulate epithelial innate response (Romanin *et al.*, 2010), induced a rise in intracellular ROS in epithelial cell ($P<0.005$).

### *Kluyveromyces marxianus* CIDCA 8154 protects against acute colitis induced by trinitrobencen sulfonic acid

To assess the ability of *K. marxianus* CIDCA 8154 to mitigate tissue damage on an acute colitis model, BALB/c mice were fed with a suspension of the yeast in drinking water. After 24 and 48 h of instillation of TNBS, animals showed a decrease in body weight (Figure 2A). Although not reaching statistical significance when compared with TNBS treated mice, animals that received the TNBS/yeast treatment had milder variations in body weight. Furthermore, histopathological analysis revealed that yeast-treated animals showed less damage in epithelium and less infiltration in the submucosa (Figure 2B). The group that received the yeast showed less overall histological damage score than those on the water group, upon challenge with TNBS (Figure 2C, $P<0.05$). These results indicate that the administration of *K. marxianus* CIDCA 8154 has a positive impact in the remission of the histopathological score. Intestinal expression of mRNA from the proinflammatory genes Ccl20, Cxcl2, Cxcl10 and IL-6 was measured by real-time qPCR at the time of sacrifice, but no significant differences were observed (data not shown). Nevertheless, circulating levels of IL-6 were consistently elevated in the TNBS-treated group, with a peak at 24 h after instillation, and were significantly reduced ($P<0.05$) upon *K. marxianus* administration at 24 and 48 h post treatment (Figure 2D).

### *Kluyveromyces marxianus* CIDCA 8154 protects from oxidative stress *in vivo*

*Caenorhabditis elegans* is a widely accepted model for the study of oxidative stress *in vivo* (Genove *et al.*, 2012), while it has been shown that the capacity of different bacterial strains to modulate oxidative stress in this system correlates with their capacity to modulate inflammation in a mouse model (Grompone *et al.*, 2012). In order to determine if exposition to yeast may also impact in the capacity of *C. elegans* to survive to oxidative stress, nematodes were fed for 7 days with *E. coli* OP50, supplemented with *K. marxianus* CIDCA 8154 live or heat-killed and as positive protection control vitamin C was used. Worms that received live yeast showed a significant increase in the survival rate compared with the control group ($P<0.001$) (Figure 3A). The protection conferred by the yeast was comparable with vitamin C, known and widely used as antioxidant. Viability of the yeast was essential to appreciate the effect, showing a correspondence with the results *in vitro*. Furthermore, to understand the mechanisms underlying the protective effect of *K. marxianus* strain, the loss-of-function *C. elegans* mutants in DAF-2, DAF-16 and SKN-1 genes were used. The insulin growth factor 1 (IGF-1) pathway is a transduction signal cascade that ultimately activates the forkhead transcription factor DAF-16, involved in the transcription of longevity and antioxidant-related genes. Oxidative stress also activates a response that is mediated by SKN-1 signalling and can extend life span independently of DAF-16 (An and Blackwell, 2003; Robida-Stubbs *et al.*, 2012). A protective effect of *K. marxianus* CIDCA 8154 in the *C. elegans* strain deficient for DAF-16 was observed ($P<0.01$), indicating that this transcriptional factor does not have a significant role on the yeast activity (Figure 3B). However, *K. marxianus* CIDCA 8154 failed to exert any protective effect on nematodes with a dysfunctional DAF-2 (the *C. elegans* insulin-receptor of the IGF-1 pathway), and SKN-1 (*C. elegans* transcriptional factor, downstream of IGF pathway) (Figure 3B). These results suggest that *K. marxianus* CIDCA 8154 modulates the SKN-1 transcription factor through the IGF pathway.

## 4. Discussion

In the last years functional food and probiotic microorganisms have received much interest from the food industry and scientific community resulting in advances on the description of their mechanisms of action on host health; however, there are still much uncertainties at the cellular and molecular level in the way that different microorganism exert their action. Among probiotics, bacterial species such as bifidobacteria and lactobacilli have been extensively studied. Although the use of probiotic yeast has been

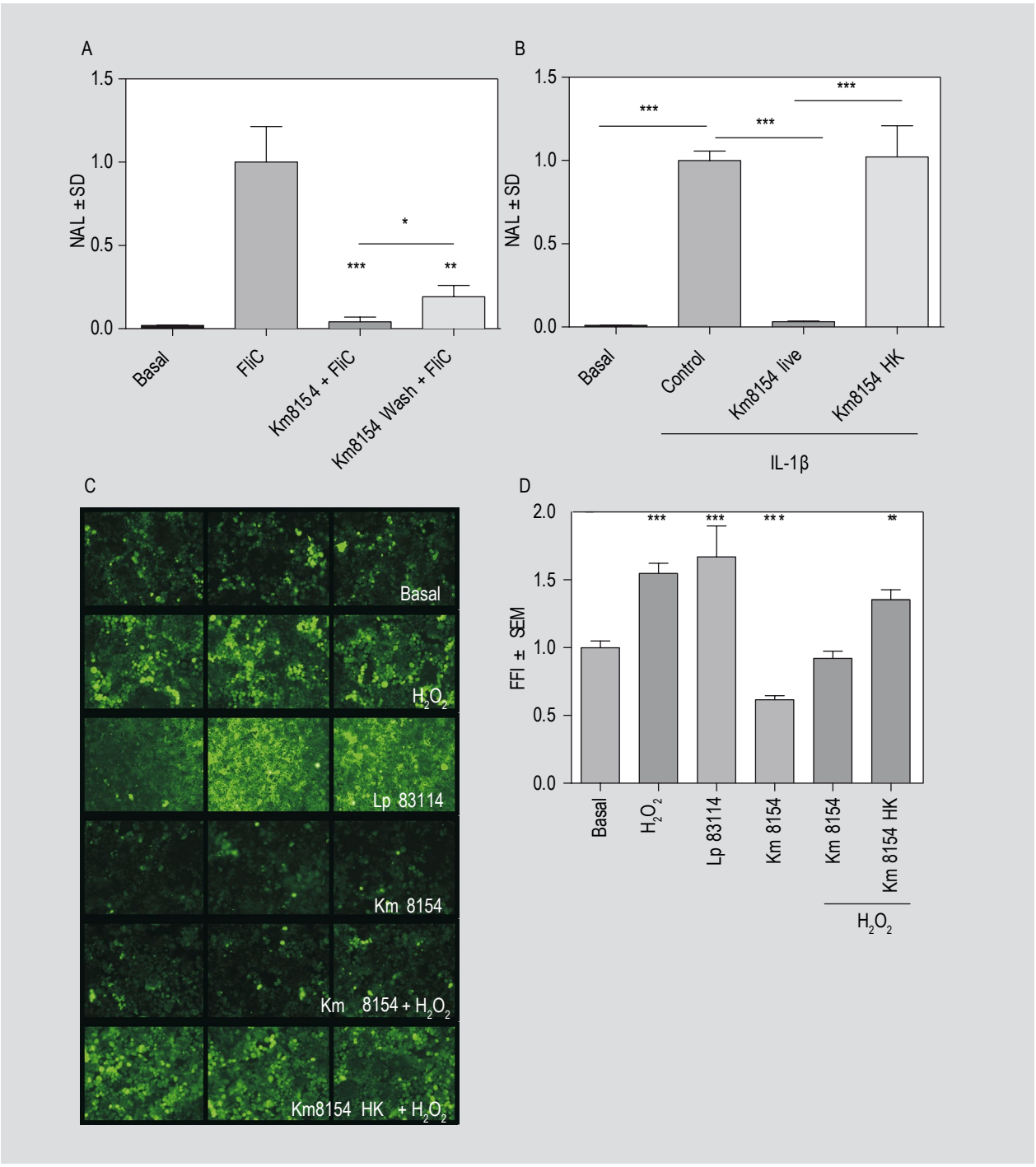



**Figure 1. *Kluyveromyces marxianus* CIDCA 8154 (Km8154) modulates intestinal epithelial inflammatory response by a reactive oxygen species (ROS) independent mechanism. (A) Flagellin (FliC) activation of reporter Caco2-CCL20-luciferase cells is abrogated by Km8154 (pairwise Student t test). Representative results of three independent experiments are depicted. (B) The capacity to modulate activation on Caco-CCL20-luciferase cells is dependent on viability of Km8154, HK (heat-killed) (pairwise Student t test). Representative results of three independent experiments are depicted. (C) Interaction with microorganisms changes redox status of epithelial cells measured by fluorescence of oxidised dichlorodihydrofluorescein dimethylacetate ester (DCFDA). $H_2O_2$ was used as positive control. Representative images are shown of three independent experiments; four different cell culture wells were used for each experiment. (D) Quantification of the mean cellular fluorescence signal (FFI; fluorescence fold increase) generated by oxidation of DCFDA shown in C (ANOVA with Dunnet post-test compared to basal condition). SEM = standard error of the mean; NAL = Normalised average luminescence; SD = Standard deviation; *** *P*<0.005; ** *P*<0.01; * *P*<0.05.**

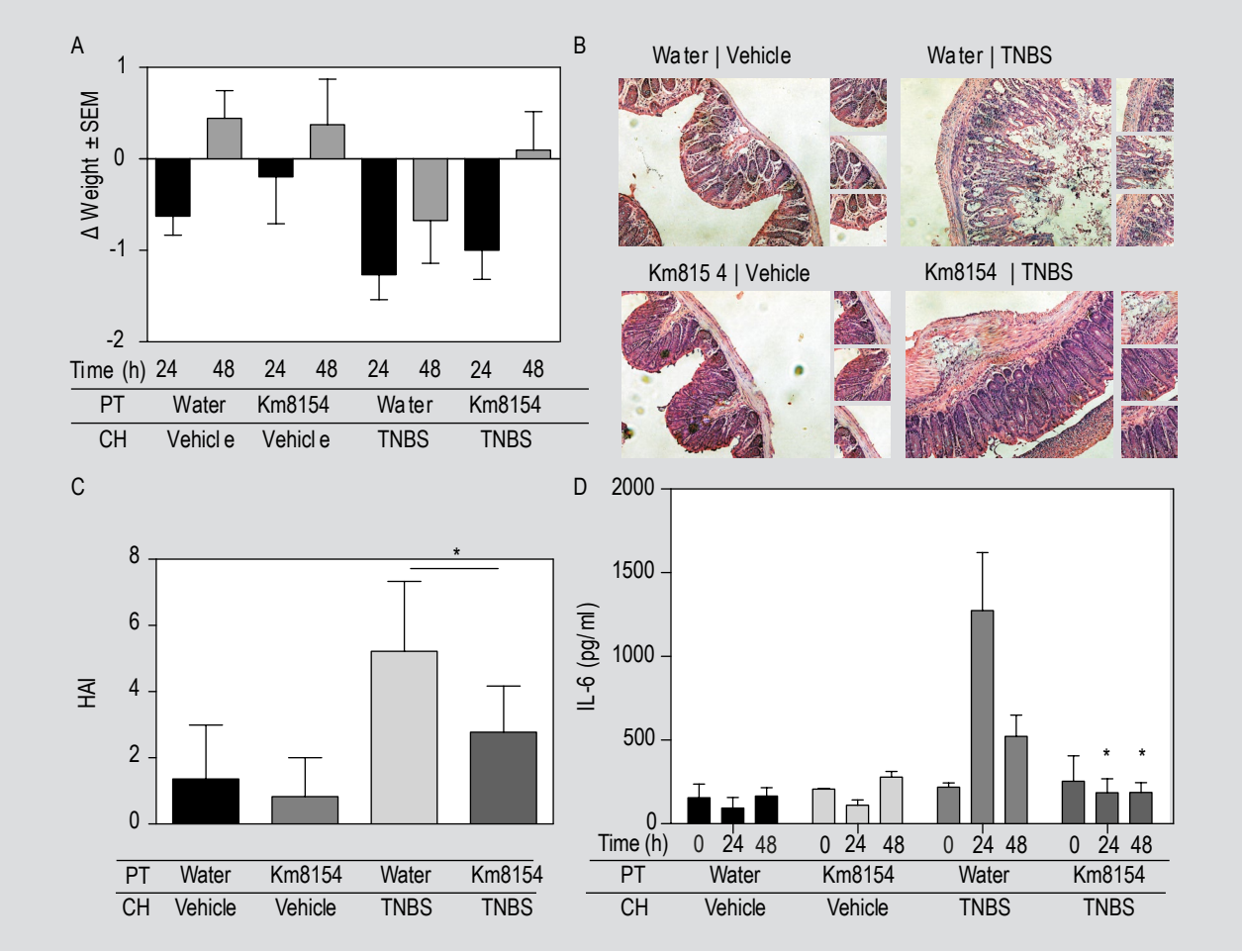
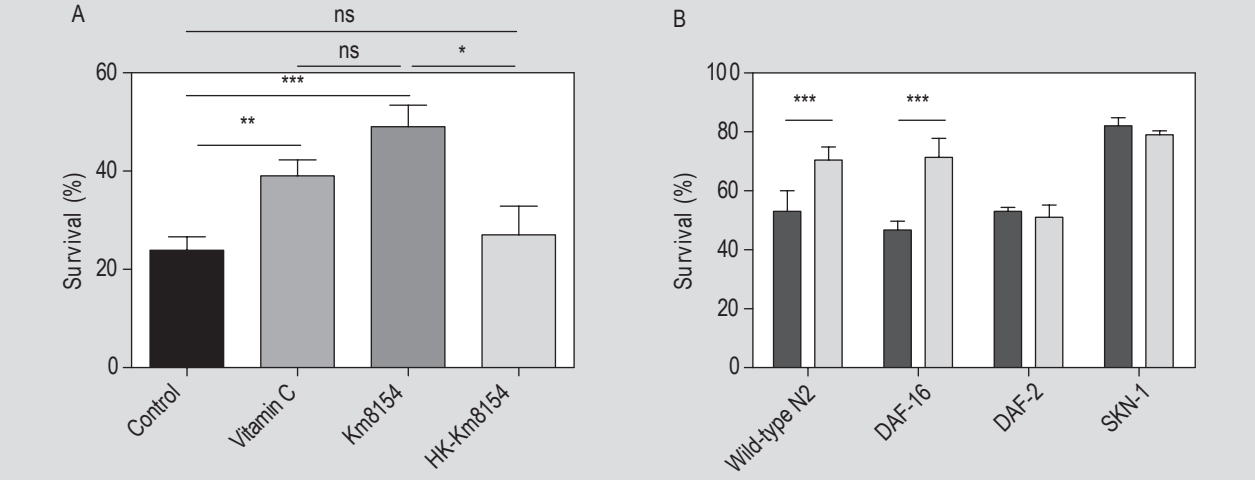



**Figure 2. *Kluyveromyces marxianus* CIDCA 8154 (Km8154) administered orally is able to protect against damage in the trinitrobencen sulfonic acid (TNBS) acute colitis mouse model. (A) Average weight change (grams ± standard error of the mean (SEM)) upon TNBS administration (n>5, representative results of three independent experiments are shown). (B) Histopathological analysis of colon indicates major damage in the TNBS group. Representative pictures of each condition are shown. (C) Histopathological activity index (HAI) calculated for each individual mouse. Mean and standard deviation (SD) are depicted (n>5, representative results of three independent experiments are shown; *P*-value indicated for Mann Whiteney U test; non-significant differences were found among other conditions by this test). (D) Serum IL-6 determination by ELISA (n>5, representative results of three independent experiments are shown; Mann Whiteney U test). PT = pretreatment; CH = challenge. * *P*<0.05.**

represented by *Saccharomyces boulardii* since several years and this is one of the few probiotics recommended for interventions in specific pathological situations by the ESPGHAN Working Group for Probiotics and Prebiotics (Szajewska *et al.*, 2014), the use and description of other probiotic yeasts has been much overlooked until the last few years (Hatoum *et al.*, 2012).

We have shown that different yeast species isolated from kefir fermented milk have the capacity to down-modulate the intestinal epithelial innate response (Romanin *et al.*, 2010). Since kefir fermentation produce a naturally low pH (3 to 4 depending on fermentation conditions, but it may drop below 2), it is not surprising that among these strains several showed a high capacity to resist low pH (Diosma *et al.*, 2014) which added to their capacity to tolerate bile acids resulted in a good capacity to survive the passage along the gastrointestinal tract. Sant'Ana *et al.* (2009) showed that *S. boulardii* displayed the greatest tolerance to simulated gastric environments compared with several *Saccharomyces cerevisiae* strains tested. We have shown that *K. marxianus* CIDCA 8154 presented a capacity to survive the passage along mice gastrointestinal tract comparable to *S. boulardii* (Diosma *et al.*, 2014). Based on its overall properties, *K. marxianus* CIDCA 8154 was selected for further characterisation. Using the widely employed TNBS model (Brenna *et al.*, 2013), we observed that *K. marxianus* CIDCA 8154 administration in drinking water was able to prevent colon inflammation. Protection was observed at the histological level and also in circulating IL-6 levels compared with the TNBS treated group (Figure 2). No differences were detected at the expression level




of several proinflammatory cytokines in colonic tissue, in concordance with similar study performed using *S. boulardii* (Grijó *et al.*, 2010). These results are in line with the few previous studies showing anti-inflammatory capacity of *K. marxianus* strains on intestinal epithelial cells (Maccaferri *et al.*, 2012; Romanin *et al.*, 2010). Recent functional studies on human dendritic cells (DCs) among more than 70 different yeast strains of different species have shown similar response patterns between *K. marxianus* and *S. boulardii* (Smith *et al.*, 2014), in spite being genetic distinct yeasts (Khatri *et al.*, 2013). We cannot conclude if the anti-inflammatory capacity of our *K. marxianus* strain shown here is due to its effect on intestinal epithelial cells and/or other cell type such as intestinal DCs.

The mechanism involved in this anti-inflammatory activity is not completely elucidated. We have shown that is dependent on cell viability, that cell contact is also important for full activity and that the modulatory effect can be observed when the yeast interacts with the apical side of enterocytes in a polarised epithelial layer (Romanin *et al.*, 2010). This is in contrast with other reports that attribute functional capacity of soluble mediators produced by yeast (Canonici *et al.*, 2011; Sougioultzis *et al.*, 2006). In concordance with Buccigrossi *et al.* (2014) that showed that *S. boulardii* can abrogate ROS production in intestinal epithelial cells, we also confirmed that *K. marxianus* is able to down-regulate ROS content in intestinal epithelial cells, which is also observed for other yeast strains of our collection (Romanin, unpublished results). It has been shown that anti-inflammatory properties of *Lactobacillus rhamnosus* GG is dependent on the induction of ROS-mediated signalling in intestinal epithelial cells which finally abrogates degradation of inhibitor κB through interference with the neddilation cascade and consequently abolishing nuclear factor κB dependent inflammatory processes (Collier-Hyams *et al.*, 2005; Kumar *et al.*, 2007; Lin *et al.*, 2009). This is also in agreement with the increased ROS production detected in intestinal epithelial cells by interaction of *L. plantarum* CIDCA 83114. In the case of the anti-inflammatory capacity of yeasts, this mechanism seems not to be involved, since the effect triggered by yeast on intracellular ROS is exactly opposite. On the other hand, there is evidence that ROS and RNS participate in tissue damage in inflammatory bowel diseases (Almenier *et al.*, 2012) and that endogenous anti-oxidative pathways are induced in these pathological conditions (Ikumoto *et al.*, 2014), being however not enough to re-establish homeostasis (Alzoghaibi, 2013). In these pathological scenarios, there is evidence that interventions aimed to reinforce antioxidative pathways contribute to attenuate the intestinal inflammation (Zhu and Li, 2012). Recently, Leblanc *et al.* (2011) and Del Carmen *et al.* (2014) showed that genetically engineered lactic acid bacteria containing ROS scavenging enzymes superoxyde dismutase (SOD) and catalase (CAT) are able to prevent colitis in a mouse model. In line with this evidence, the ROS scavenging capacity of yeast shown here could be involved in its anti-inflammatory activity in the colitis model. This capacity may be explained by the expression of CAT and SOD enzymes in *K. marxianus* (Dellomonaco *et al.*, 2007; Koleva *et al.*, 2008; Pinheiro *et al.*, 2002). The successful use of probiotic yeast as treatment for intestinal inflammatory diseases such as rotavirus infection has been previously demonstrated to be dependent of modulation of ROS production (Buccigrossi *et al.*, 2014). Moreover it was described that genetically modified *Lactobacillus casei* engineered to produce CAT or SOD were able to revert the symptoms induced by intrarectal TNBS administration in mice, while the wild type strain ($CAT^-$ and $SOD^-$) did not protect (LeBlanc *et al.*, 2011).

The use of *C. elegans* as model organism for unravelling innate immunity circuits is gaining acceptance due to its inherent handling simplicity (Marsh and May, 2012) Recently, Grompone *et al.* (2012) showed an interesting correlation between the capacity of lactic acid bacteria to dampen oxidative stress in a *C. elegans* model with the anti-inflammatory properties of these strains using *in vitro* or *in vivo* models similar to the ones used here. Among 78 lactic acid bacterial strains screened in a *C. elegans* oxidative stress model, authors selected the strain that have the most

potent capacity to prevent acute oxidative stress-induced death and confirmed that this strain has powerful anti-inflammatory capacity tested either on a TNBS-induced colitis model or in *in vitro* tests. Furthermore, authors determined that in *C. elegans* the insulin/IGF-1-like signalling pathway (IIS) signalling proteins DAF-2/DAF-16 are responsible for the protective effect elicited by the lactic acid bacteria. The effects of these bacteria involved also the SKN-1 transcription factor. These pathways are also necessary for the beneficial effects exert by other probiotic bacteria on *C. elegans* (Komura *et al.*, 2013) and they has also been described as target of Celecoxib (Celebrex®), a non-steroidal anti-inflamatory drug (Ching *et al.*, 2011). The effects of Celecoxib required the activity of DAF-16, a transcription factor suggesting an important role of IIS pathway as molecular target of anti-inflammatory compounds. In the case of yeast, we were able to use the *C. elegans* model to study probiotic yeast-host interaction, validating this powerful system to dissect the mechanisms of this interaction. We could determine that *K. marxianus* CIDCA 8154 is able to protect *C. elegans* from acute oxidative stress and that this property is dependent on yeast viability, furthermore this activity is dependent on DAF2 receptor and SKN-1 transcription factor but independent of DAF16. These results show that the protective capacity shown in this model is not dependent exclusively on yeast properties, since it also needs a signalling activity on *C. elegans* through the activation of the transcription factor SKN-1 via IGF-1 pathway. In *C. elegans* SKN-1, which is related to vertebrate Nrf proteins, promotes expression of detoxification enzymes in response to oxidative stress (An and Blackwell, 2003). Either in the case of lactic acid bacteria or the yeasts, it is not yet known which microbial ligand(s) trigger the DAF2 pathway. However, it is evident that both types of microorganisms use different downstream pathways, since DAF16 transcription factor is dispensable for *K. marxianus-C. elegans* interaction. Further studies are needed to completely dissect the pathways involved in the anti-oxidative action of the probiotic microorganisms on this model.

## 5. Conclusions

The probiotic yeast *K. marxianus* CIDCA 8154 is able to protect from TNBS-induced colitis and also in an acute oxidative stress model in *C. elegans*. The pathways involved in these effects seem to be different in the case of this probiotic yeast than for lactic acid bacteria. This evidence provide a rational for the formulation of combined probiotic products containing microorganisms that trigger different anti-inflammatory mechanisms that could be a useful intervention complement in gastrointestinal pathologies that involve inflammatory mechanisms.

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