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Forkhead Box Protein O1 is Linked to Anti-Inflammatory Probiotic Bacteria Acting through Nuclear Factor-κB Pathway

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Abstract

Probiotics are widely used to promote health benefits around the world. Nevertheless, the mechanisms whereby probiotics exert its beneficial effect on the host are not well elucidated yet. In an attempt to obtain relevant insights on probiotics mechanisms of action, we studied the probiotic response via Nuclear factor-κB (NF-κB) and Forkhead box protein O1 (FoxO1), two transcription factors that were previously related with probiotic effects. We performed *in vitro* analysis to activate these transcription factors with Tumor Necrosis Factor alpha (TNFα) and Hydrogen Peroxide (H_2O_2) stimuli using a set of probiotic strains co-cultured with HT-29 cells. We found three strains, LrBPL8, LcA1 and LaBPL71 capable to reducing the NF-κB activation pathway in an inflammatory context. We also found that LcA1 reduced FoxO1 activation while another strain, IPM C+, increased it after the hydrogen peroxide treatment under the same conditions. Moreover, we described a complex relationship between FoxO1 downstream gene expression and these anti-inflammatory strains. Our results show that more than one pathway could be targeting NF-κB modulation, indicating the complexity of the probiotics' mechanisms of action. The *in vitro* data presented here may help to design multi-strain probiotics mix that take advantage of the complementary and synergistic effects that they may induce in the host.

Introduction

It is well known that the use of probiotics for the prevention of a variety of diseases is becoming more and more popular around the world. The Food and Agriculture Organization of the United Nations and the World Health Organization define probiotics as "live microorganisms which when administered in adequate amounts, confer a health benefit on the host" [1]. Among bacteria, Lactobacilli and Bifidobacteria are the main genera of probiotics which have been implicated in beneficial outcomes. Probiotics can exert health benefits for treatment and prevention of gastrointestinal disorders, such as impairment of colonic transit, enteric infections and post-antibiotic syndrome, reduction of recurrence of *Clostridium difficile* associated diarrhea, prevention of necrotizing enterocolitis and colorectal cancer, and prevention and treatment of inflammatory bowel diseases [2-7].

Some possible mechanisms involved in the beneficial effects of probiotics are the interaction with other microbes, the improved functions of the gut epithelium and interactions with innate defense cells [8]. Although the mechanisms are well described *in vitro* and in animal models, the actual mechanism of action of probiotics in humans has not been clearly addressed [9]. Meta-analysis of human studies using probiotics interventions have generated contradictory results in part due to differences in study design (variety of probiotic strains, daily doses and length of administration) but also due to the poor understanding of the mechanisms by which these probiotics elicit their effects [10-13].

In animal models, two transcription factors have been involved in the effect of the probiotics: Nuclear factor-κB (NF-κB) and Forkhead box protein O1 (FoxO1). The NF-κB family of transcription factors play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival being a key regulator between probiotics and intestinal epithelial cells [14,15]. Usually, NF-κB is inactive in the cytoplasm; when pro-inflammatory stimuli trigger signaling pathways, the inhibitor molecule IκB is phosphorylated, targeting it for ubiquitination and consequent proteasomal degradation. Once unbound from IκB, NF-κB is able to migrate into the nucleus, bind target promoters and activate the transcription of effector genes [16]. Probiotics have shown to either prevent or promote NF-κB activation, acting in different steps of this signaling pathway like stabilizing IκB-α or activating RelA subunit [17-19].

The transcription factor FoxO1 is a member of the mammalian forkhead box O class subfamily that regulates a wide array of cellular processes, including the cell cycle, apoptosis, proliferation, survival, metabolism, DNA repair, response to oxidative stress, differentiation and homeostasis of stem/progenitor cells [20]. In its non-phosphorylated state, FoxO1 is located in the nucleus, where it is transcriptionally active and up-regulates gene expression involved in growth arrest, response to oxidative stress and apoptosis [21]. Conversely, phosphorylation of FoxO1 by Akt causes its release from DNA, binding to 14-3-3 protein

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and rapidly relocating from the nucleus to the cytoplasm, where it becomes inactive [21]. It has been described that *C. elegans* FoxO1 homologous, daf-16, has been involved in the anti-inflammatory and anti-oxidant effect of the *Lactobacillus rhamnosus* CNCM I-3690 strain [22].

In order to better understand the implication of these transcription factors in the mechanism of action of different probiotic strains, we carried out a series of studies with 10 potential probiotic strains, analyzing the implication of NF-κB and FoxO1 in the response to oxidative and pro-inflammatory stimuli. We identified three bacterial strains that reduced the NF-κB activation in response to TNFα. We further studied the link between FoxO1 and the NF-κB observed responses, finding dissimilar behavior in the three studied strains.

Materials and Methods

Reagents

Unless otherwise indicated, all chemicals used were of the highest available grade and purchased from Sigma Aldrich (St. Louis, MO, USA). Culture media, fetal bovine serum (FBS), lipofectamine and consumables for cell culture were obtained from Life Technologies (Carlsbad, CA, USA), GE Healthcare (Waukesha, WI, USA), and Greiner Bio-one (Frickenhausen, Germany). The GFP-FoxO1 vector was donated by Domenico Accili (Addgene plasmid #17551) [23].

Cell Lines and culture conditions

HT-29 (ATCC HTB-38) cells were cultured in DMEM medium and supplemented with 10% (v/v) FBS. HT-29-NF-κB-hrGFP clon E5 cells were cultured in RPMI1640 medium and supplemented with 10% (v/v) FBS. Cells were routinely propagated in 25 or 75 cm^2 tissue culture flasks at 37ºC, 5% CO$_2$ in a humidified incubator until reaching approximately 70% confluence [24]. Subsequently, cells were trypsinized and concentration was adjusted for different experimental settings. In all described assays, cells were cultured for less than twenty passages.

Bacteria and culture conditions

Probiotic bacteria were kindly provided by Biopolis SL; the strains used were *Lactobacillus casei* A1 (LcA1), *Lactobacillus rhamnosus* BPL8 (LrBPL8), *Lactobacillus rhamnosus* BPL15 (LrBPL15), *Lactobacillus plantarum* BPL7 (LpBPL7), *Lactobacillus fermentum* BPL34 (LfBPL34), *Lactobacillus acidophilus* PBL71 (LaBPL71), *Bifidobacterium longum* BPL001 (BlBPL001), *Bifidobacterium longum* BPLA4 (BlBPLA4) and *Streptococcus thermophilus* BPL67 (StBPL67). Control strains (IPM C+ and IPM C-: *Lactobacillus rhamnosus* strains) were used throughout the experiments. All bacteria were cultured in Man Rogosa and Sharpe Media (MRS) (Oxoid, Basingstoke, UK), under anaerobic conditions generated by AnaroGen (Oxoid, Basingstoke, UK). Bacteria were subcultured twice for 24 h and centrifuged for 5 min at 3000 g, the bacterial pellet were washed once with phosphate buffered saline (PBS) and resuspended for quantification. Live bacterial concentration was assessed by flow cytometry using Accuri C6 (BD Biosciences, San Diego, CA, USA) equipment. A 100 µL fraction of bacterial dilution was acquired and the number of living bacteria was recorded using propidium iodide staining. Afterwards, counts were verified by plating different dilutions of the bacterial suspension.

Reporter gene assay

HT-29-NF-κB-hrGFP clone E5 cells were seeded in 96-well plates

(5 × 10⁴cells/well). After 24 h, bacteria (multiplicity of infection [MOI] of 100 or 50) were added to each well and incubated for 2 h. After the co-culture period, gentamicin (50 µg/mL) and TNF-α (1 ng/mL) were added. Cells were further incubated for 24 h. Non-treated cells (basal) and cells treated only with TNF-α or bacteria were included as controls.

Lastly, cells were trypsinized and resuspended to perform flow cytometry analysis. Cells were analyzed using an Accuri C6 flow cytometer equipped with 488 and 640 nm lasers. BD Accuri C6 Software V1.0.264.21 was used for data acquisition and analysis. The GFP and propidium iodide fluorescence emissions were detected using band-pass filters 533/30 and 585/40, respectively. For each sample, 5000 counts gated on an FSC versus SSC dot plot (excluding doublets) were recorded. Only single living cells (those that excluded propidium iodide), were considered for analysis.

FoxO1 localization

HT-29 cells were seeded in 12-well plates (3 × 10⁵ cells/well). Twenty-four hours after plating, cells were transfected with FoxO1-GFP plasmid using Lipofectamine 2000 following manufacturer's protocol. Briefly, 1 µg of supercoiled plasmid and 5 µl Lipofectamine per sample were each diluted in independent 100 µl volumes of Opti-Mem medium and incubated for 5 min at room temperature. Subsequently, both solutions were mixed and incubated for 30 min at room temperature to allow the formation of Lipofectamine: DNA complexes. Mix was added to each well and removed 24 h later.

To study FoxO1 location under oxidative stress conditions, H$_2$O$_2$ treatment was applied. Cells were co-cultured with an MOI of 100 bacteria per cell. After 2 h, gentamycin (50 µg/mL) and 500 µM of H$_2$O$_2$ were added for 2 h. For TNF-α stimulus, co-cultures were performed at an MOI of 100; the TNF-α (1 ng/mL) treatment started 2 or 6 h after co-culture and gentamicin was added at 2 h of co-culture. Cells were further incubated for 22 h.

For both treatments, cells were washed twice with PBS, fixed with PFA 4% in PBS and nuclei were stained with TO-PRO-3® dye (Thermo Fisher, Waltham, MA, USA). The slides were observed under confocal microscope DMI6000, TCS-SP5 (Leica, Wetzlar, Germany) using 488 nm laser excitation for GFP and 633 for TO-PRO®-3 and the subcellular location of FoxO1 was recorded in 100 cells per slide. Images were captured with an x63 oil objective and an open pinhole to collect fluorescence from the entire depth of the cell.

The percentage of FoxO1 activation was calculated as the percentage of cells with fluorescence in the nucleus plus half of the percentage of cells with fluorescence both in the nucleus and in the cytoplasm.

Expression of FoxO1 downstream genes

HT-29 cells were co-cultured with bacteria in 24-well plate with addition of gentamicin (50 µg/mL) 2 h later. After 24 h of co-culture, cells were washed twice with PBS and RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions and measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific, NY, USA). After checking the absence of genomic DNA contamination by qPCR, copy DNA (cDNA) was synthetized starting from 500 ng of total RNA using random hexamers and SuperScript II Reverse transcriptase (Thermo Scientific, NY, USA).

The qPCR reactions were performed using 5 µL SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), equimolar amounts of forward and reverse primers (1 µM; Integrated DNA Technologies, Coralville, IA, USA) and 1 µL cDNA in a final volume

of 10 µL. Samples were analyzed in duplicate in the Eco Real Time PCR System (Illumina, San Diego, CA, USA). Standard amplification conditions were as follow: 3 min at 95°C, 45 cycles of 10 s at 95°C and 30 s at 60°C. After determining that the efficiency of the primers were comparable and near 100%, the delta cycle threshold (CT) value was calculated from the difference in the CT of the gene of interest and that of the β-Actin gene (NM 001101.3) Data are presented as $2^{-\Delta\Delta CT}$ [25] (Supplementary Table 1).

The oligonucleotide used for amplification is described in detail in Supplementary Table S1.

Statistical Analysis

For the reporter gene assay, data was normalized against TNF-α (considered as 100%) and basal (considered as 0%) controls. Statistical differences between individual samples were defined using ANOVA followed by the Bonferroni t test for multiple comparisons. For FoxO1 activation assay, data was normalized against the untreated cells (0%), and Student's t test was applied for individual comparison of different treatments. For gene expression analysis, data was analyzed by the Mann-Whitney test. All the data were plotted as the mean (± SEM) of triplicates from independent experiments. Statistical analysis was completed using GraphPad Prism V 5.00 software (GraphPad Software, La Jolla, CA, USA).

Results

NF-κB modulation

To better understand the anti-inflammatory impact of the potential probiotic strains on human cells *in vitro*, we followed NF-κB activation upon TNFα stimulation using a reporter gene assay. We first studied NF-κB activation by the bacteria in an inflammatory or non-inflammatory context. Without TNFα, none of the bacteria modulated NF-κB activation (Figure 1). Cells treated with 1 ng/µl of TNFα and probiotics showed a wide variety of responses. When co-cultured with a MOI of 50, cells treated with LrBPL8 and IPM C+ showed a reduction of NF-κB activation of 17.2% and 16.0% respectively (p<0.01) (Figure 1C). At MOI 100, the percentage of modulation was higher for these strains (61.2% for LrBPL8 and 42.7% IPM C+) (p<0.001) whereas strains LcA1 and LaBPL71 were able to reduce the NF-κB activation in a 32.9% and 56.6% respectively (p<0.001).

Interestingly, several bacteria produced an increase in the activation of NF-κB in an inflammatory context. Strains LrBPL15 (p<0.05), LfBPL34 (p<0.01), BlBPLA4 (p<0.001) and StBPL67 (p<0.001)
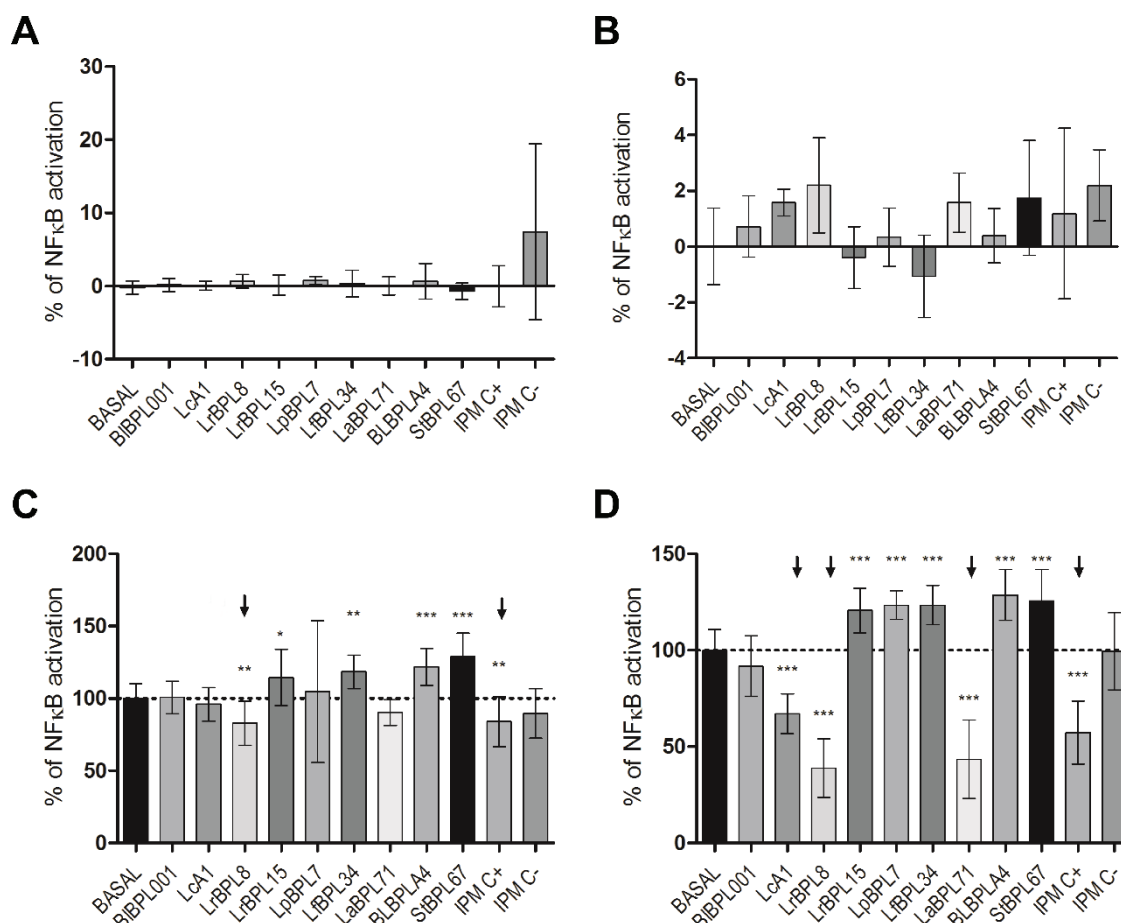

Figure 1: Identification of strains that modulate activation of NF- κB in response to TNFα. HT-29-NF-κB-hrGFP E5 clone cells were co-cultured with lactic acid bacteria and bifidobacteria strains at a multiplicity of infection of 50 (A, C) and 100 (B, D) bacteria per cell. After 2 h, antibiotic (A, B) and antibiotic and TNFα (1 ng/ml) (C, D) were added. 22 h later, NF-κB activation was assessed by the percentage of GFP+ cells by flow cytometry. Data was normalized against TNF-*α* (control: considered as 100) and basal cells (without TNF-*α*, considered as 0). Arrows shown the strain and conditions where anti-inflammatory properties were observed. Results are shown as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

activated NF-κB between 14.6% and 28.9% when used in a MOI of 50. When MOI 100 was used, the same strains and also LpBPL7 were able to activate NF-κB between 20.6% and 28.8% (p<0.001).

Activation of FoxO1 in response to oxidative stress and TNFα stimuli

Having found the three anti-inflammatory strains with the NF-κB reporter assay, we attempted to elucidate the relationship of these bacteria and the FoxO1 pathway. Previous finding from our group demonstrated that FoxO1 homologous gene in *C. elegans*, daf-16, is involved in the protection exerted by probiotics from oxidative stress induced by H_2O_2 in this organism [22]. Based on these findings, we wanted to explore if this transcription factor was also related to the

oxidative stress response in mammalian cells. For that, translocation of FoxO1 to the nucleus was assessed under confocal microscopy as a marker of this protein activation. Three different localization of FoxO1 were observed in the cells; i) cells with cytoplasmic exclusive fluorescence; ii) cells with fluorescence mainly in the nucleus, and iii) a group of cells with similar fluorescence levels in both nucleus and cytoplasm (Figure 2). Treatment of cells with H_2O_2 for 2 h produced the activation of 13.9% of FoxO1 when compared to untreated cells (p<0.01). Moreover, the addition of the bacteria to the cells also produced the activation of FoxO1. Strains LrBPL8 and LaBPL71 had no effect upon stimulation of intestinal epithelial cells with H_2O_2. On the other hand, LcA1 had a protective effect, noticed by the reduction of FoxO1 activation (p<0.05), and IPM C+ significantly increased FoxO1 translocation to the nucleus (p<0.05).
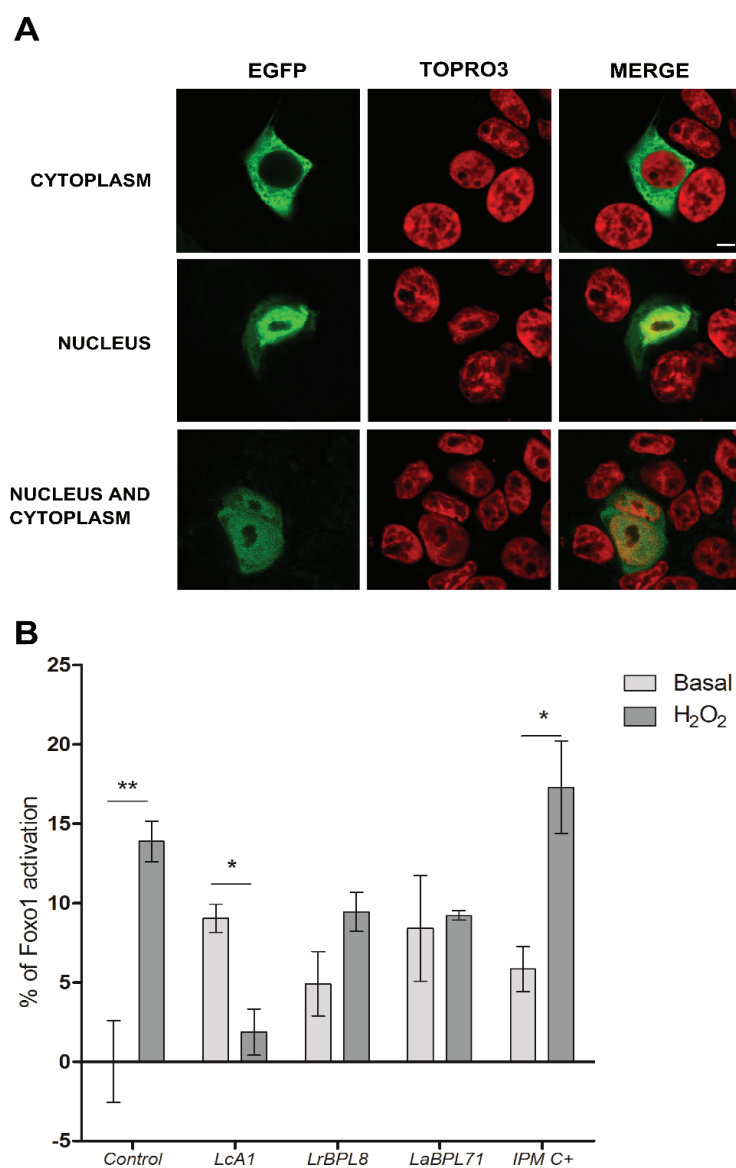
Figure 2: Intestinal epithelial cells response to oxidative stress modulated by probiotics. HT-29 cells were transfected to express FoxO1 fused to GFP and observed under confocal microscope. A: Representative images of cytoplasmic, nuclear and both compartment locations. B: Location of FoxO1 under treatment with 500 μM of hydrogen peroxide and bacterial strains. The activation of FoxO1 was calculated as the percentage of cells with nuclear fluorescence plus half of the percentage of cells with FoxO1 in both compartments. Nucleus was stained with TO-PRO3. Data was normalized to the untreated cells (Control=0).
Scale bar=5 nm
Results are shown as the mean ± SEM. * p<0.05, ** p<0.01

Furthermore, we wanted to explore the pattern of activation of FoxO1 in the response of the strains in an inflammatory context. Treatment of the cells with TNFα significantly reduced the FoxO1 activation compared to untreated cells (control). After probiotic treatment, there is an increase in FoxO1 activation (Figure 3). When both TNFα and probiotics are added, the levels of activation return to the unstimulated level. Although all the tested probiotics showed the same tendency, the differences between the co-cultured cells alone and the co-cultured cells plus TNFα differ significantly only for LcA1 and IPM C+ (p<0.05) (Figure 3).

Expression of FoxO1 related genes

We determined the level of expression of five genes downstream to FoxO1 (CAT, SOD2, G6PC, DDB1 and GADD45a) and two interacting genes (PPARγ and SIRT2) to further explore the scope of FoxO1 activation (Figure 4).

Catalase (CAT) expression had a significant increase for both LcA1 and IPM C+ strains (p<0.05), being higher in the case of LcA1 (1.8 vs. 1.2-fold increase) (Figure 4). The opposite was confirmed for SOD2, were IPM C+ produced a 5-fold increase while LcA1 produced a 2-fold increase (p<0.05). All the tested strains produced an increase in the expression level of G6PC gene (p<0.05) (between 1.8 and 3.0 fold increase) and no effect was observed in the DDB1 gene expression level.

All the strains, except for IPM C+ increased the level of PPARγ and GADD45α transcription (1.9 to 3.0-fold for PPARγ and 1.4 to 2.1-fold for GADD45α) (p<0.05). No increase in the level of SIRT2 expression was observed with the tested strains, nonetheless the control strain increased the expression of this gene 2.2-fold (p<0.01).

Discussion

Accurate assessment of the mechanism involved in the probiotic response is essential for probiotic development and application. In this study, we propose a polyfunctional exploration of diverse transcription regulatory pathways in order to better understand probiotic strains mechanisms of action.

We used an NF-κB reporter cell line in order to identify probiotics with potential anti-inflammatory activity. This approach allowed us to determine three strains able to reduce NF-κB activation in an inflammatory context. All of them showed a dose dependent behavior, which highlights the relevance of determining the adequate amounts of probiotic for further treatments.

Probiotic effects together with commensal microbes are crucial in barrier integrity and mucosal homeostasis in different body sites like the skin or the gut, among others [26,27]. Probiotics and commensals can antagonize against pathogens through direct, i.e., bacteriocin secretion or indirect, i.e., immune signaling, mechanisms to contribute to gut and skin barrier homeostasis. Probiotic effect could be elicited by secreted molecules, indeed, administration of the isolated bacterial-derived metabolites or molecules may be sufficient to promote the desired effect [28]. *Lactobacillus salivarius* UCC 118 secretes Abp118, a class II bacteriocin which effectively inhibits *L. monocytogenes* infection and barrier disruption *in vivo* in a mouse [29]. More recently, bacteriocins secreted by some probiotics have shown the capability to modulating immune responses through NF-κB signaling [30]. It has been shown that microbial colonization can stimulate innate and adaptive immune responses in the intestinal mucosa, mediating an indirect pathogen antagonism [31]. Probiotics can stimulate the production of epithelial β-defensins and antimicrobial C-type lectins in the gut by stimulating innate receptors expressed in the intestinal mucosa [26]. Interestingly, some skin antimicrobial peptides interact directly with Toll Like Receptors (TLRs) to inhibit inflammation or act as potent chemotactic signals attracting monocytes, dendritic cells, neutrophils and T cells (Supplementary Figure 1) [32]. In our study, we observed diverse levels of NF-κB activation by our Lactobacilli strains, suggesting that different
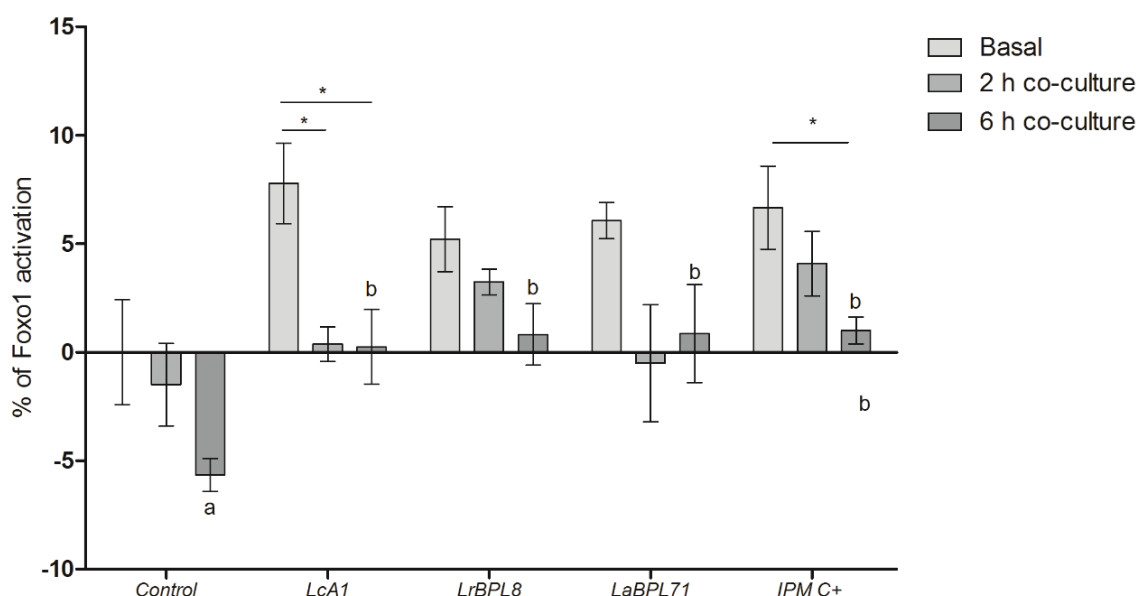
Figure 3: Pro-inflammatory stimulus reduces FoxO1 activation in intestinal epithelial cells independently of the presence of probiotic strains. HT-29 cells were transfected to express FoxO1 fused to GFP and observed under confocal microscope. Probiotics were added to the transfected cells except for the control group. After 2 or 6 h of co-culture, 1 ng/ml of TNFα was added and incubated during 22 h; basal condition does not have TNFα addition.
Results are shown as the mean ± SEM. Values with different letters are significantly different *p<0.05
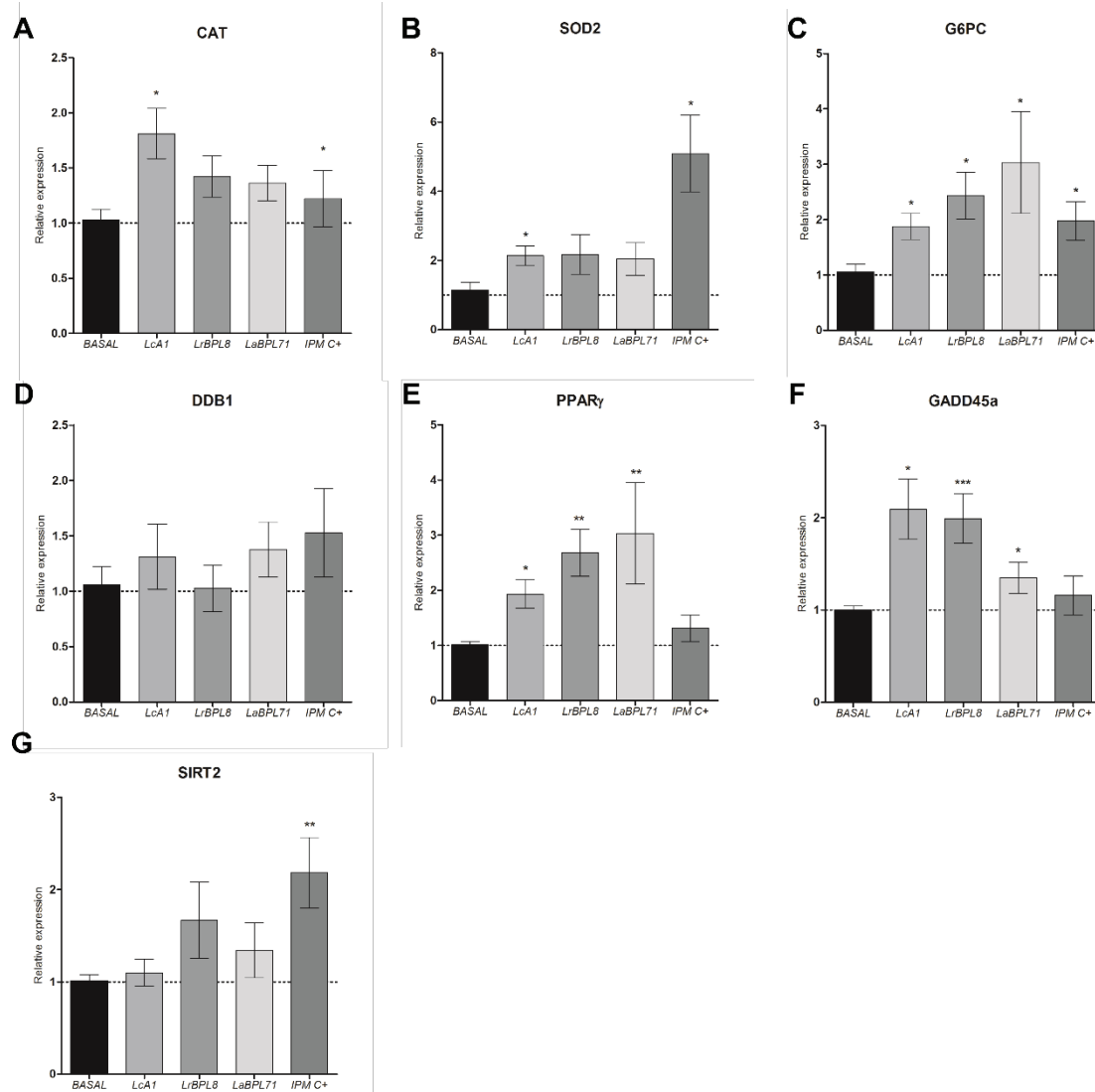
Figure 4: Probiotic strains trigger specific pathways related to FoxO1 signaling. HT-29 cells were co-cultured with probiotic strains during 24 h and gene expression was analyzed using qPCR. Significant differences are relative to the basal condition without bacteria. The expression level of each gene was normalized to that of β-actin. The results are shown as the mean ± SEM.
*p<0.05, ** p<0.01, ***p<0.001

mechanisms could be involved to explain such differences. In future work, it would be interesting to analyze whether the effect of NF-κB activation level is related or not to specific secreted molecules such as bacteriocins.

Moreover, *in vitro* analysis of probiotic inter-strain variability is crucial in order to choose specific strains for particular uses. Four *Lactobacillus rhamnosus* strains were included in this assay and all of them exhibited different behavior; for instance, with a MOI of 100, two strains were anti-inflammatory, one did not have an effect, and the other showed a pro-inflammatory behavior. This intra-species variation had been widely reported before [33,34]. Here, we provide new insights for probiotic identification and selection before designing treatments or probiotics based products.

The JNK/FoxO signaling pathway is a crucial pathway against oxidative stress [35,36]. For this reason, we evaluated the relationship between these anti-inflammatory bacteria and the oxidative stress response through FoxO1 activation. The H_2O_2 treatment produced a predictable increase in the FoxO1 translocation to the nucleus. The increase of FoxO1 activation by the co-culture with the anti-inflammatory strains could be attributed to the increase in oxidative stress levels produced by the bacteria metabolism residues in culture medium. When both, the oxidative and the probiotic stimuli converge in the cell, LcA1 seems capable of reducing oxidative stress as suggested by the decrease of FoxO1 activation; however, since IPM C+ increases FoxO1 activation, the drop shown by LcA1 could be attributed to limited activation of FoxO1 to prioritize the activation of more essential pathways.

Our results showed that the stimuli with TNFα could inactivate FoxO1 in our experimental conditions; suggesting cells seem to prioritize the immune response over the oxidative or damage response.

Different responses to TNFα were observed for FoxO1 in diverse models, for instance, treatment of pulmonary artery smooth muscle cells with TNFα produce down regulation of FoxO1; in bone marrow derived mesenchymal stem cells TNFα also inhibits FoxO1. Nevertheless, in adipocytes and fibroblasts the treatment with TNFα activates FoxO1 [37-39]. Our observations are aligned with previous findings that show an activation of AKT pathway in response to TNFα in HT-29 cells.

In multiple cell types, FoxO transcription factors have a protective role in resistance to oxidative stress through regulation of the antioxidant genes SOD2 and CAT, as well as additional cell survival pathways. Under conditions of starvation or oxidative stress, FoxO can be activated by increasing AMPK activity or decreasing AKT activity [40]. In this work, the strains LcA1, LrBPL8, LaBPL71 and IPM C+ co-cultured with HT-29 cells produced the activation of FoxO1. Nevertheless, downstream genes activated in each case are very dissimilar, suggesting that the related activated pathways are also strain-specific. FoxO transcription factors have cofactors that consist in other transcription factors that co-regulate each other [41]. The presence or absence of these cofactors can explain why the activation of FoxO1 does not have the same output in different probiotic strains.

Both LcA1 and IPM C+ produced an increase in the expression of genes involved in the protection of oxidative stress. Nevertheless, the pathways involved in this response are diverse for each strain. While IPM C+ produces an increase in the expression of SOD2, LcA1seems to exert its oxidative effect through the CAT pathway.

The PPARγ gene codifies for a nuclear hormone receptor than can regulate intestinal inflammation and homeostasis, and it has been previously reported as a target for probiotics [42]. This protein inhibits both NF-κB and FoxO1 activity [42,43]. For LcA1, LrBPL8 and LaBPL71 this could be the pathway involved in NF-κB modulation.

In addition, FoxO1 represses PPARγ in a SIRT2 dependent manner [44]. This could be the mechanism employed by IPM C+ were activation of SIRT2 is observed while the expression of PPARγ remains the same as the control.

The transcription factor FoxO1 has been related to the response of probiotics in *C. elegans* oxidative stress, skin acne and alcoholic fatty liver [22,45,46]. Nevertheless, this is the first study linking FoxO1 activation to probiotics response in intestinal mammalian cells. It has been previously shown that NF-κB pathway is linked to survival pathways including PI3-kinase/AKT, one of the main FoxO regulatory pathways, and it has been shown that AKT activates NF-κB transcription factors [47-49]. In addition, FoxO3a can inhibit NF-κB activation, reducing downstream production of inflammatory cytokines, and reduction of FoxO levels can determine the increase in the level of inflammatory cytokines through NF-κB activation [50,51]. As this family of transcription factors is connected with several immune pathways, we consider that the study of FoxO1 activation in response to probiotics could be a powerful tool to dissect the inflammatory response mechanisms.

Conclusion

The results presented here show the complexity of the probiotics' mechanisms of action. Treatment with anti-inflammatory strains resulted in different FoxO1 responses and the activation of a different set of FoxO1 downstream genes for each strain. These observations evidence the intervention of more than one pathway that could be targeting the same output, in this case the NF-κB modulation. Our findings support the necessity to combine multiple strain probiotic

products, based on the potential complementary and synergistic effects that they could induce on the host. Furthermore, *in vitro* data is crucial for probiotic characterization but considering the complexity of our data, *in vivo* studies are mandatory to better confirm the involved pathways.

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