

ORIGINAL ARTICLE

Transduction pathways regulating the trophic effects of *Saccharomyces boulardii* in rat intestinal mucosa

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Abstract

Saccharomyces boulardii is a probiotic yeast that is widely prescribed in lyophilized form; it determines several effects in human and rat small intestine including endoluminal secretion of enzymes and of polyamines, stimulation of microvillous enzymes, of sIgA, increased production of the receptor for polymeric immunoglobulins by crypt cells, and enhanced D-glucose uptake. **Aim.** The objective of this study was to determine the pathway(s) by which these effects generated by the yeast are transduced into mucosal cells. **Methods.** Litters of six growing Wistar rats each were treated with *S. boulardii* (50 µg/gram body weight) or with saline between days 30 and 34 postpartum. For each animal, the cytosol was prepared from the whole mucosa after the fat cake was discarded. Several known intestinal substrates were immunoprecipitated and immunoblotted using specific antibodies recognizing the non-, mono-, or diphosphorylated forms of these substrates. The signals were detected using Echochemiluminescence (ECL) and were measured by optodensitometry. **Results.** Treatment with *S. boulardii* markedly enhanced the RAS-GAP-RAF-ERK_{1,2} pathway with participation of growth receptor bound 2 protein, SHC, SOS, and CRKII. Unit p85α of phosphatidylinositol 3 kinase, tested in its phosphorylated form, was also enhanced by the probiotic compared to control samples. In rats treated with an inhibitor of RAF-1 and of ERK_{1,2} (PD098059) the expression of mucosal disaccharidases was inhibited by about 50%. **Conclusion.** The probiotic *S. boulardii* generates *in vivo* mitogen and metabolic signals that are transduced into intestinal mucosal cells, downstream from the apical membrane to the nuclei, using recruitment substrates and serine, threonine, or tyrosine kinases.

Key Words: D-Glucose uptake, disaccharidases, polyamines, probiotic, *Saccharomyces boulardii*, transduction pathways

Introduction

Saccharomyces boulardii is a biotherapeutic agent effective in acute and chronic enterocolopathies [1–6]. Lyophilized preparations of *S. boulardii* (Biocodex, Gentilly, France) when administered orally to humans or rats exert trophic intestinal effects including increases in the specific and total activities of microvillous enzymes such as sucrase [7], aminopeptidase [8], trehalase [9], enhanced secretion of s-IgA in jejunal and ileal fluid [10], increased production of the receptor for polymeric immunoglobulins by crypt cells [10], and a marked stimulation of the sodium-dependent D-glucose uptake by brush border membrane (BBM) vesicles

with a corresponding increase in the sodium D-glucose co-transporter 1 (SGLT-1) [11]. These effects, at least in part, are mediated by the endoluminal release of polyamines [12], as yeast cells contain substantial amounts of spermine and spermidine [12,13]. In addition, yeast cells secrete enzymes such as sucrase [7], α,α-trehalase [9], leucine-aminopeptidase [8], and a novel protein phosphatase that inhibits *Escherichia coli* O₅₅B₅ endotoxin by dephosphorylation into the intestinal lumen [14].

The objectives of the present study were to identify the transduction pathways by which these effects generated by *S. boulardii* are regulated in rat intestinal mucosal cells.

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(Received 29 April 2009; accepted 31 October 2009)

ISSN 0036-5521 print/ISSN 1502-7708 online © 2010 Informa UK Ltd.
DOI: 10.3109/00365520903453141

Materials and methods

Media and culture conditions

Saccharomyces boulardii cells were inoculated in YPD (yeast extract, 0.5%; peptone, 2%; glucose, 2%; DIFCO, Detroit, MI, USA) media and grown at 30°C with moderate shaking [7] in a lyophilized form (100 mg/flask, biologic activity, 2.9×10^9 viable cells/ml) by the manufacturer (Biocodex, Gentilly, France).

Animals and treatments

The present study was approved by the Animal Welfare Committee of the Catholic University of Louvain. It was conducted according to the APS's Guiding Principles in the Care and Use of animals. Litters of growing Wistar rats were reduced to six pups per lactating mother to equalize conditions of nursing and feeding. Four groups of six animals each were studied between days 30 and 34 postpartum corresponding to the post weaning period of growth. *S. boulardii* was administered to rats ($n = 12$; 100 mg/flask, biologic activity, 2.9×10^9 viable cells/ml) at a dose of 50 µg of lyophilized yeast cells per gram body weight per day in 0.1 ml saline by nasogastric intubation, twice daily [7]. Control groups ($n = 12$) were treated according to the same schedule and received equal volumes of saline. PD098059, an inhibitor of mitogen activated protein kinase kinase (MAPKK) and of ERK_{1,2}, was purchased from Santa Cruz Biotech (CA, USA). The inhibitor was administered intraperitoneally, twice daily [15] at the dose of 2 µg per gram body weight per day from days 30–34 postpartum. Control rats were treated according to the same schedule and received equal volumes of the vehicle.

Collection of tissues

On the day of study, rats were killed by decapitation, and the small intestine from the pylorus to the ileocecal valve was immediately excised. The total length was measured and divided into two equal segments. The proximal half was considered the jejunum and the distal half the ileum. Both segments were opened longitudinally, were rinsed in cold NaCl 0.9% and the mucosa was scrapped off between glass slides. After weighing, it was wrapped in Parafilm® paper and frozen in liquid nitrogen (–170°C) until use.

Enzymes assays

Disaccharidase activities were measured on jejunal mucosal samples using the micromethod of Messer

and Dahlqvist [16]. One unit equals 1 µmol of glucose formed per minute and per gram protein. Protein content was determined by the method proposed by Lowry et al. [17].

Immunoprecipitation and immunoblotting

Intestinal mucosal samples from *S. boulardii*-treated rats and controls were centrifuged and the fat cake (supernate) was discarded. The clear cytosol was adapted to get 100 mg protein in each sample; it was thereafter diluted fivefold (1/5) with immunoprecipitation buffer (RIPA: TRIS 0.025 M, triton-X100 0.5%, Non-idet P40 0.5%, pH 7.4) containing antiproteases (10 µl) including pepstatin, leupeptine, and phenylmethylsulfonylfluoride (PMSF). To each sample, 100 µl of protein A Sepharose 4B, diluted in RIPA buffer (1/1), was added and mixed by rotation for 4 h at 4°C. Thereafter, the samples were centrifuged at 24,000 g for 5 min at 4°C. The pre-cleared supernates were collected and mixed with 10 µl immunoglobulin G (IgG) purified antibodies by rotation overnight at 4°C. Then, 100 µl of protein A sepharose 4B was added to the samples and mixed by rotation for 4 h at 4°C. The sepharose beads were washed twice with RIPA buffer and once with TRIS, 10 mM buffer. After the last wash, 5 µl bromophenol blue (Invitrogen, Carsbas, CA, USA) diluted in 10 µl aqua milli Q was added to the beads, the supernates having been discarded. Immunoprecipitation and immunoblotting were performed using the one-step complete IP-Western kit (Genescript Corporation, Piscataway, NJ, USA). This novel procedure allows the detection of nanograms of antigen by echochemiluminescence (ECL) without showing co-immunoprecipitation of the heavy and light chains of the IgG antibody.

Antibodies

Unless otherwise indicated, all antibodies used were polyclonal rabbit antibodies purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Negative controls were performed using polyclonal, non-specific antibodies in place of the specific antibody.

The specific antibodies recognized intestinal substrates or tyrosin-serine-threonine kinases, demonstrated by previous studies, to be present in rat small bowel mucosal extracts [15,18]. The antibody used for growth receptor bound 2 protein (GRB2) was affinity purified and raised against a peptide (C-23) mapping the C terminus of GRB2 of rat origin (p21). The antibody used for Sarc homology 2 domain containing-transforming protein C1 (SHC)

recognized the diphosphorylated form (tyr 239, tyr 240) of the three proteins p46, p52 and p66 encoded by the gene. The antibody against RAS-GAP was raised against amino acids 171–448 of RAS-GAP of human origin. Concerning p70 S6 kinase, we used an affinity purified antibody raised against the double phosphorylated form (thr 421, ser 424) of the C-terminal peptide of rat p70S6 kinase. Extracellular signal regulated kinase 1 and 2 (ERK_{1,2}) were tested by antibodies raised against the non-phosphorylated form of ERK_{1,2} and the double phosphorylated form, that is, a short amino sequence containing phosphorylated thr 202 and tyr 204 for ERK₁ and thr 185 and tyr 187 for ERK₂ of human origin. The protein serine, threonine kinase (RAF-1) is a MAPKK that was assessed by a purified antibody raised against a short amino acid sequence containing the double phosphorylated form (thr 508 and ser 601) of RAF-1 of human origin. To identify CRKII, we used an affinity purified rabbit polyclonal antibody raised against a peptide mapping the C terminus of CRKII of human origin. The antibody raised against phosphatidylinositol 3 kinase (Pi-3 kinase) was used at 1/20 and 1/40 dilutions and recognized the p110, p85 α , p85 β , p55, and p50 units of the complex enzyme. To clearly demonstrate the effect of *S. boulardii* on Pi-3 kinase, we also used an affinity purified antibody raised against a short amino acid sequence containing the phosphorylated form (tyr-508) of α -p85 regulatory unit of human origin. p38 MAP kinase was tested using an affinity purified antibody raised against a phosphopeptide including isoforms p38 β , γ , and δ , with sequences surrounding thr-183, tyr-185 of p38 γ , thr-180, tyr-182 of p38 β , and thre-180, tyr-182 of p38 δ . The transcriptional motif (p65) of heterodimeric nuclear factor kappa B (NFKB) was studied in controls and *S. boulardii*-treated rats using a polyclonal antibody raised against a polypeptide (aa 1–286) of NFKB of human origin, which contains the transcriptional activator motif (p65). The other transduction factors studied were the β subunit of the insulin receptor, the phosphorylated form of protein kinase C (PKC), phospholipase C-isoenzyme-gamma-1 (PLC-J1), the integrin receptor tyrosine kinase, and protein kinase B (PKB).

Statistical analysis

All experiments were made in duplicate or triplicate. The intensity and amount of signals were measured by optodensitometry, using arbitrary units of optical density (OD) with adjusted volume: OD $\times$ mm² and area. All optodensitometric measurements were made more than 4 times on each Western blot and were

subjected to statistical analysis ANOVA, and Neuman's Keul for multiple statistical comparisons, if the *F* value was correct. Under other conditions, we used the non-parametric test of Mann-Whitney *U* test. Differences between means were considered statistically significant for *p* < 0.05. The same procedure was applied for mucosal mass parameters and enzymes activities. All data are means $\pm$ standard deviation (SD). Standard deviation less than 5% of the mean are not depicted.

Results

Figure 1 (upper panel, left side) shows the signals of Pi-3 kinase subunits obtained from mucosal extracts of *S. boulardii*-treated rats (A+ at 1/20 and 1/40 dilutions) and controls (B- at 1/20 and 1/40 dilutions). It identifies four units: the catalytic unit p110 (weak signal), the two regulating units p85 α and p85 β isoforms, and p55/p50 proteins that appear in one band and have previously been evidenced in rat brain, muscle, liver, and intestine [18]. As shown in Figures 1 and 2, the signals of Pi-3 kinase units were much more marked in *S. boulardii*-treated rats than in controls. This is especially evident in lane A+, dilution 1/20, and in the corresponding optodensitometric data shown in Figure 2 for p85 α , p85 β , and p50/55. The p110 signal was too weak for interpretation. These units co-immunoprecipitated because our antibody was raised against the entire NH₂ – SH₂ domain of Pi-3 kinase, which is common to p85 α , p85 β , p55, and p50 [18]. To further confirm that the signal of *S. boulardii* is regulated by p85 α , we used an antibody recognizing the tyrosine phosphorylated form (tyr 508) of p85 α . Figure 3 demonstrates that the signal of the p85 α phosphorylated form was much more intense in *S. boulardii*-treated rats (OD $\times$ mm²: 27.75 $\pm$ 1.1; area: 24.13 $\pm$ 0.9) than in controls (OD $\times$ mm²: 14.09 $\pm$ 0.9, *p* < 0.02; area: 14.1 $\pm$ 0.5, *p* < 0.02).

Growth receptor bound 2 protein (p21) was detected in both groups as a strong signal (Figure 1) but was only slightly increased (OD $\times$ mm²: 18.57 $\pm$ 1.3 vs 16.71 $\pm$ 1.1) (area = 16.99 $\pm$ 0.7 vs 15.70 $\pm$ 0.2, NS) in the group treated with the probiotic. It is a small cytoplasmic protein containing two SH₃ and one SH₂ domains [19]. Growth receptor bound 2 protein interacts in response to extra-membrane stimuli and binds to mammalian SOS (mSOS not shown here) and SHC via its SH₂ domain [19]. Isoforms (p52, p46) of SHC, when phosphorylated [20], function as initiators of the RAS signaling cascade after their interaction with the complex GRB2-SOS. As shown in Figure 4, we found

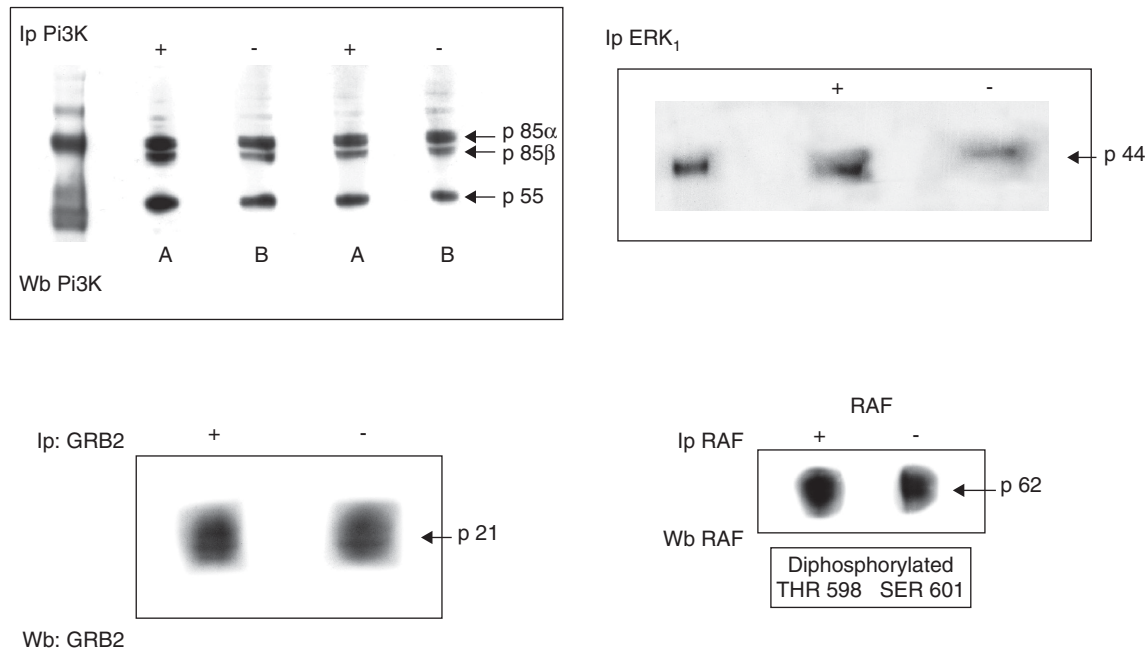


Figure 1. Four immunoblots revealing changes in Pi-3 kinase (upper left), ERK₁ (upper right), GRB2 (lower left), and diphosphorylated RAF (on threonine 598, serine 601) (lower right) in *Saccharomyces boulardii*-treated rats (+) and controls (-). In Pi-3 kinase blot, the two first lanes on the left (A+ and B-) were tested at a dilution of 1/20, while the two lanes on the right (A+ and B-) were assayed at a dilution of 1/40.

diphosphorylated SHC on tyr residues 239/240 (p52, p46) in mucosal extracts from treated and control rats; but the SHC signal of *S. boulardii*-treated rats was much more important; the same is true for the p21 associated protein, which is probably GRB2. In addition, the complex Son of sevenless (SOS) and GRB2 interact with CRKII, another adapter protein that bind to GRB2, though their SH2 domains. In our study, CRKII was also increased by 1.5-fold in response to *S. boulardii* treatment (Figure 5) compared to controls.

As in target cell lines stimulated *in vitro* with growth factors, we have detected a 120-kDa protein identified as GAP (the mean GTPase-activating protein of the normal form of p21 RAS) in intestinal mucosal extracts from weaned rats (day 36) (Figure 6). In animals treated with *S. boulardii*, immunoprecipitation and Western blotting of mucosal extracts with an anti-RASGAP polyclonal antibody (reacting with a large protein sequence of human RASGAP corresponding to amino acids 171–443 that include two SH₂ domains and one SH₃ domain) revealed a major signal of ~ 120 kDa (GAP) ($OD \times mm^2$: 9.47 ± 0.12 ; area: 8.77 ± 1.08), a weak signal at ~ 60 kDa (likely p62 SRC) ($OD \times mm^2$: 5.95 ± 0.7 ; area: 6.64 ± 0.9), a marked signal of 44 kDa (likely ERK_{1,2}) ($OD \times mm^2$: 11.9 ± 1.2 ; area: 14.82 ± 0.16), and a massive protein of ~ 21 kDa (p21, likely RAS) (Figure 6). Interestingly, in control rats, p120 GAP was virtually not detected while p60 was very weak ($OD \times mm^2$:

2.27 ± 0.3 ; area: 2.63 ± 0.86 , $p < 0.05$) and p44 markedly decreased ($OD \times mm^2$: 3.22 ± 0.8 ; area: 3.04 ± 1.06 , $p < 0.01$) indicating that their interactions with p120 GAP are physiologically relevant and represent a direct binding between these proteins. The massive signal of p21 (RAS) was only slightly increased in *S. boulardii*-treated rats ($OD \times mm^2$: 57.56 vs 53.71 in controls) but the other proteins were much more increased in the treated group (Figure 5).

In mucosal extracts of *S. boulardii*-treated rats as well as in control rats we identified the phosphorylated form of RAF-1 (thr 598, ser 601) (Figure 1), a serine, threonine kinase (MAPKK) involved in mitogenic signals from the membrane to the nucleus. Diphosphorylated RAF was clearly increased in *S. boulardii*-treated rats ($OD \times mm^2$: 50 ± 0.2 ; area: 32 ± 0.1) compared to control ($OD \times mm^2$: 37 ± 0.01 treated $n = 6$ vs 22 ± 0.6 controls $n = 6$, $p < 0.05$) (Figures 1). Extracellular signal-activated kinases 1 and 2 are mitogenic activated protein kinases (MAPKs) that are cytoplasmic and intranuclear, acting as intermediates between the activated cascade of RAS-GAP-RAF-1 and the response of eukaryotic cells to extracellular signals. Extracellular signal-activated kinases can be directly activated on cell surface by endoluminal stimuli and once phosphorylated, migrate into the nucleus [21]. Their normal precursor is RAF-1. As shown in Figure 1 (upper right panel) the signal of ERK₁ was enhanced by 3 times in *S. boulardii*-treated rats ($OD \times mm^2$: 15 ± 0.2 ;

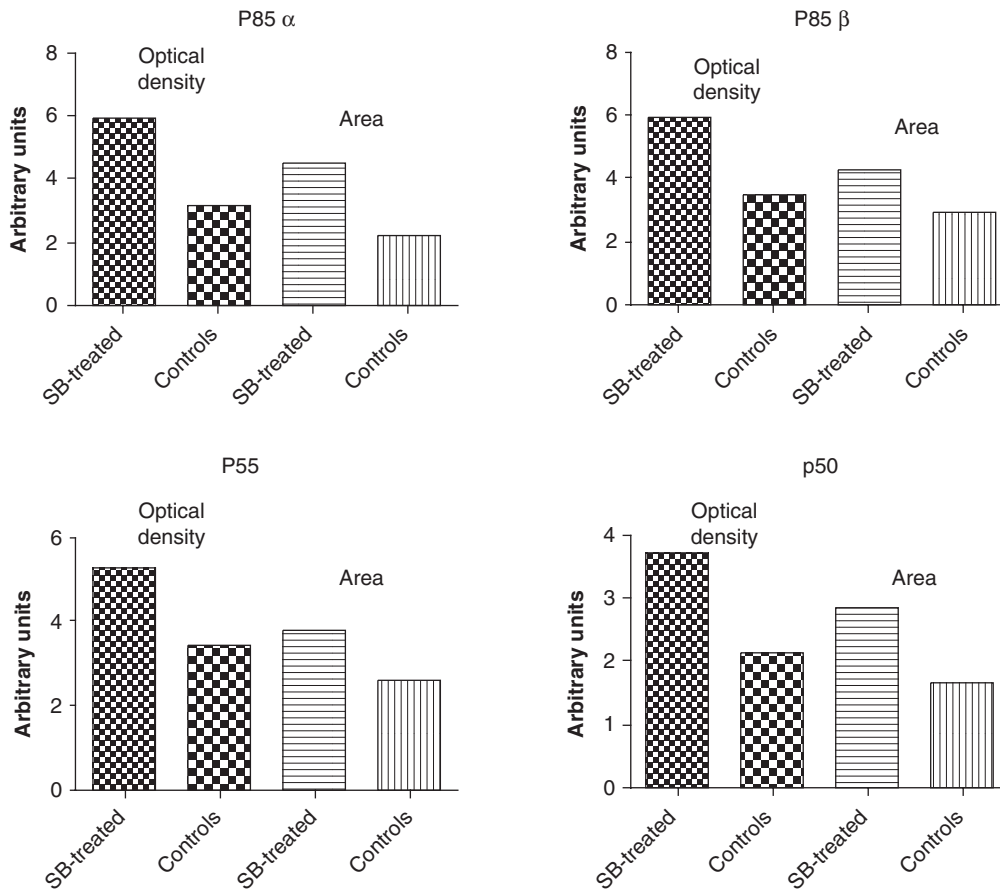


Figure 2. Optodensitometric data (OD in arbitrary units adj. vol. $\times$ mm² and area) measured for the subunits p85 α , p85 β , p55/p50 (lower half of the surface area of the signal) in *Saccharomyces boulardii*-treated rats and controls. The signal of p85 α and β phosphorylated were much more intense in *S. boulardii*-treated rats (OD $\times$ mm²: 6.5 ± 1.1 ; area: 4.8 ± 0.9 ; $n = 6$) than in controls (OD $\times$ mm²: 4.5 ± 0.9 , $p < 0.02$; area: 2.1 ± 0.5 , $n = 6$, $p < 0.02$). GRB2 p(21) was detected in both groups as a strong signal but was only slightly increased in *S. boulardii*-treated rats (OD $\times$ mm²: 18.57 ± 1.3 vs 16.71 ± 1.1 , $n = 6$, NS) (area: 16.99 ± 0.7 vs 15.70 ± 0.2 , $n = 6$, NS).

area: 17 ± 6.2) compared to controls (OD $\times$ mm²: 5.8 ± 0.09 ; area: 5.7 ± 0.08 , $p < 0.02$). To further demonstrate the regulation by ERKs we used an antibody recognizing the diphosphorylated forms of ERK₁ and of ERK₂. As shown in Figure 3 (left panel), ERK₁ and ERK₂ were detected as a large aggregated complex that was more marked in *S. boulardii*-treated rats (OD $\times$ mm²: 41.5 ± 0.5 ; area: 39.6 ± 0.22) than in controls (OD $\times$ mm²: 28.3 ± 0.3 ; area: 23.4 ± 0.14 , $p < 0.01$). The signals were fused because molecular weights of ERK₁ and ERK₂ are very close and there is more than 90% homology between the structures of the two serine-threonine kinases. To further provide evidence that the ERK₁/ERK₂ pathway mediates the signals generated by *S. boulardii*, we treated weaned rats (days 30–34 postpartum) interperitoneally with PD098059, a specific inhibitor of MAPKK and in turn of ERK₁ and ERK₂. The inhibitor was administered by intraperitoneal injection at a low dose (2 μ g/g body weight twice daily) (median of lethal dose = 200 μ g/g) for 4 days, 1 h before the

administration of *S. boulardii*. Control rats received the vehicle of PD098059. Growing rats treated with PD098059 showed the same weight gain as did the control group without change in final intestinal length. However, as shown in Table I, mucosal mass, whether jejunal or ileal mucosa, was significantly lower ($p < 0.015$, $n = 16$) by –11 to –13% in the experimental group compared to the control group. Changes in the specific activity of disaccharidases are detailed in Table II. Compared to controls, disaccharidase activities (lactase, sucrase, maltase-glucoamylase) were decreased by –41 to –51% in the PD098059 group. Protein content remained equivalent in both groups.

In quiescent cells, p90 RSK is a serine, threonine kinase that can form a complex with either ERK₁ or ERK₂. Its activation by trophic factors is followed very rapidly by the phosphorylation of p70S6 kinase, a ribosomal enzyme. Both enzymes are serine, threonine phosphorylated in response to mitogen stimulation. Figure 6 (left panel) shows that p90 RSK and p70 S6 kinase were associated together

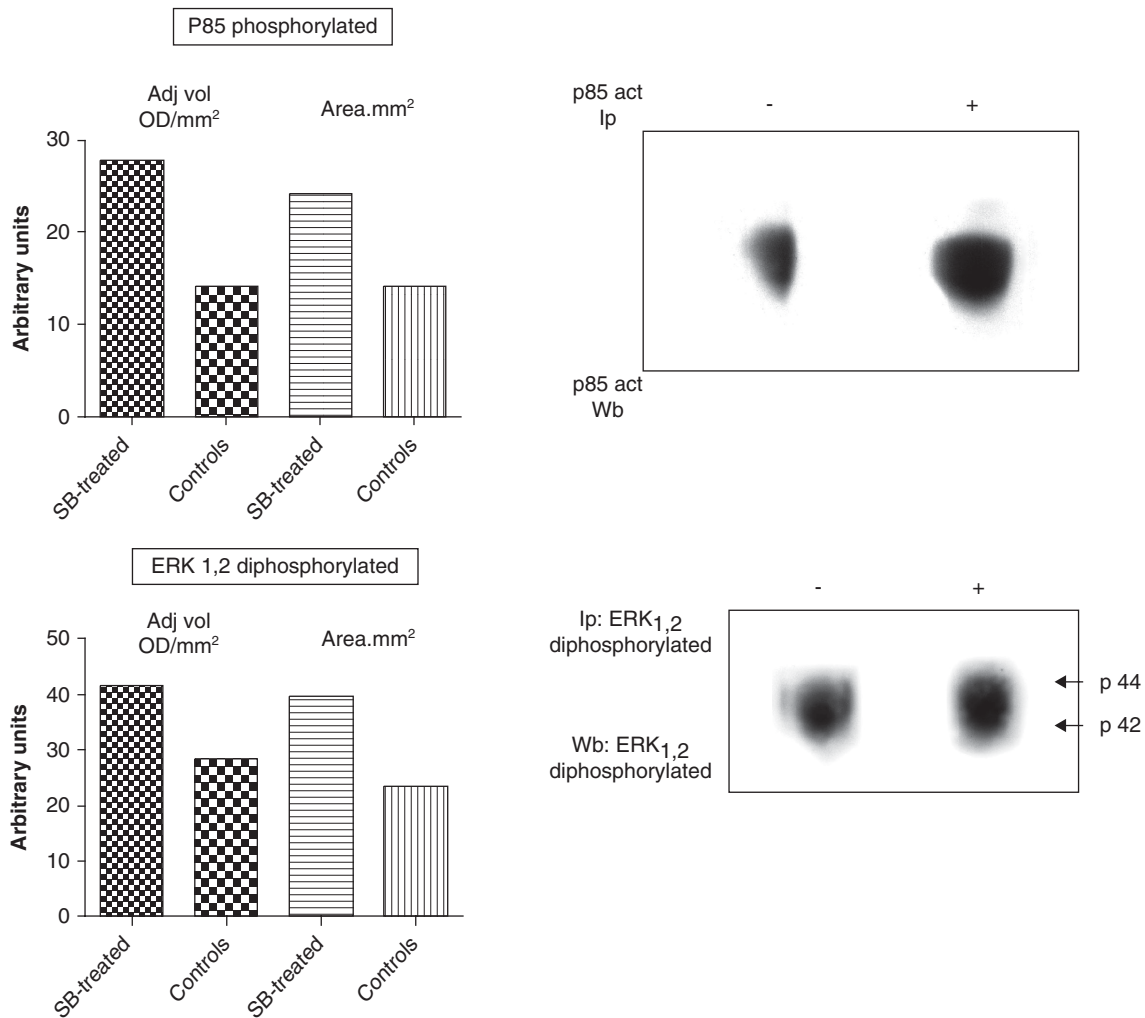


Figure 3. Upper graphs: (right) immunoblot of the phosphorylated form of p85a (on threonine 508) in mucosal extracts from *Saccharomyces boulardii*-treated rats (+) and controls (-). Lower graphs: (right) immunoblot of the diphosphorylated forms of ERK₁ and ERK₂ in *S. boulardii*-treated rats (+) and controls (-). The corresponding optodensitometric data include for ERK₁ and ERK₂ diphosphorylated: OD $\times$ mm² = 42 $\pm$ 0.7 vs 27 $\pm$ 0.7, n = 6, p < 0.01; and for p85 diphosphorylated OD $\times$ mm² = 25 $\pm$ 1.3 vs 11 $\pm$, n = 6, p < 0.01.

and with other proteins including p44/p42 and p22. The former are likely ERK₁ and ERK₂. The signals of these enzyme-proteins were only slightly increased in *S. boulardii*-treated rats compared to controls. On the other hand, we observed no changes between experimental and control groups in the expression of integrin receptor tyrosine kinase, phospholipase-C isoenzyme gamma, protein kinase B, and the tyrosine kinase domain of the insulin receptor. Figure 7 shows (on the left side) the signals of NF κ B and of p38 MAP kinase and the corresponding optodensitometric data (on the right side). NF κ B (p65) was not detected in *S. boulardii*-treated rats while a large signal was detected in controls. This was associated with marked differences in OD $\times$ mm². In concordance, the signal of p38 MAP kinase was found to be slightly decreased in *S. boulardii*-treated rats.

Discussion

There is so far no information regarding the mechanism(s) by which the probiotic *S. boulardii* generates mitogenic and metabolic effects that are transduced from the apical epithelial membrane downstream to the nucleus. Our study shows that intestinal mucosal samples from intact rats as well as from rats treated with *S. boulardii* express several signaling substrates, recruited proteins, serine, threonine, and tyrosine kinases that transduce mitogenic and metabolic signals from the epithelial membrane to the nucleus. These signaling substrates were detected in our previous studies [15,18] and in the present one using specific antibodies acting on mucosal extracts from both treated and control rats that were equivalent in terms of age, weight, treatment schedules, nutrition,

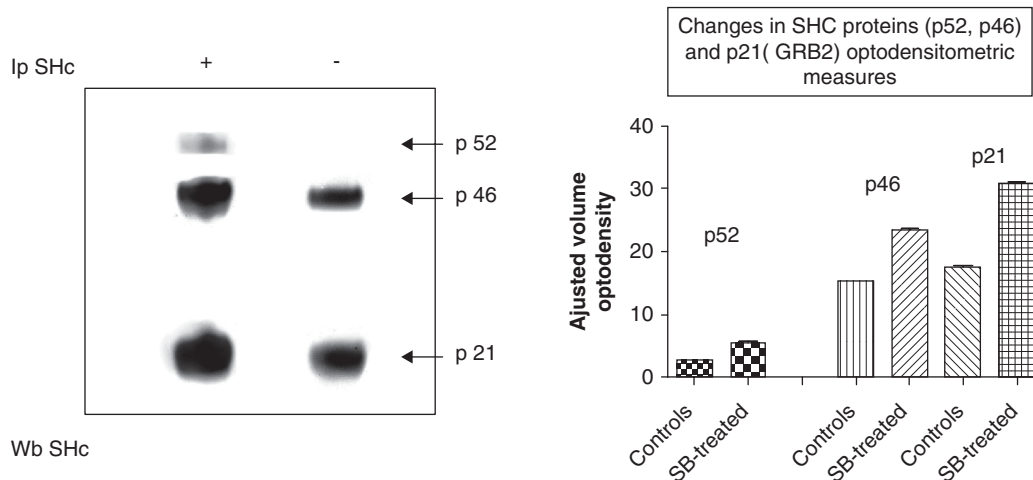


Figure 4. On the left: immunoblot showing SHC (p52, p46; p66 was not detected) and p21 (likely GRB2) in *Saccharomyces boulardii*-treated rats (+) and in controls (-); on the right: p52 ($OD \times mm^2 = 8.77 \pm 0.1$ vs 1.5×0.5 , $n = 6$, $p < 0.02$); p46 ($OD \times mm^2 = 24.3 \pm 0.2$ vs 15 ± 0.2 , $n = 6$, $p < 0.01$); p21 ($OD \times mm^2 = 30 \pm 0.3$ vs 19 ± 0.1 , $n = 6$, $p < 0.01$).

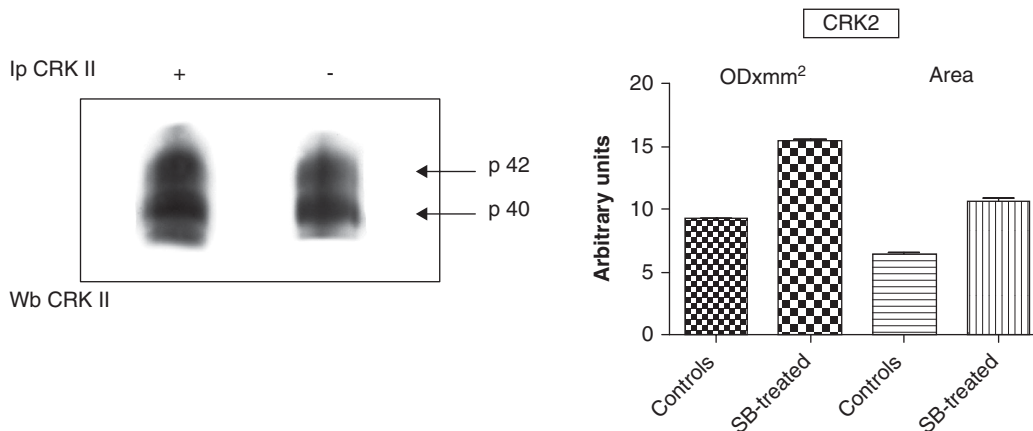


Figure 5. On the left: immunoblot showing CRKII in *Saccharomyces boulardii*-treated rats (+) and controls (-); on the right: corresponding optodensitometric data. For CRKII in *S. boulardii*-treated rats the mean value of $OD \times mm^2$ measured in six rats was 15 ± 0.2 vs 9.2 ± 0.5 in control rats ($n = 6$, $p < 0.05$).

and protein concentration. Results concerned epithelial cells because in a preliminary experiment on isolated enterocytes similar differences between ERK₁ and ERK₂ were observed (data not shown). However, because we used whole mucosal extracts, we cannot exclude the inclusion of other cell types such as lymphocytes, fibroblasts, macrophages, and so on. First of all, the non-phosphorylated (Figure 1) and the diphosphorylated forms of ERK₁ (p44) and ERK₂ (p42) were unequivocally increased in *S. boulardii*-treated rats compared to controls (Figure 3). This prompted us to confirm their role in the transduction of trophic signals by inhibiting their activation, especially because these key enzymes down-regulate mitogenic and metabolic stimuli from the epithelial membrane to the nucleus. The administration of PD098059 to *S. boulardii*-treated

rats at low doses (2 μ g/g), clearly inhibited both mucosal mass (-11% to -13% compared to controls) (Table I) and the expression of BBM disaccharidases (-41% to -51% vs controls) after 96 h (Table II) [15,22]. ERK_{1,2} (p44,p42) are extracellular signal regulated kinases activated by extracellular stimuli that migrate downstream into the cytoplasm and nuclei [21] where they are involved in both initiation and regulation of meiosis, mitosis, and post-mitotic functions of differentiated cells by phosphorylating a number of nuclear transcription factors namely ELK-1 (ETS family, transcription activators that play a role in regulating epithelial cell differentiation and proliferation) [21,23] and EIF4EBP1 (eukaryotic translation initiation factor 4E-B binding protein-1 required for initiation of translation). On the other hand, our study

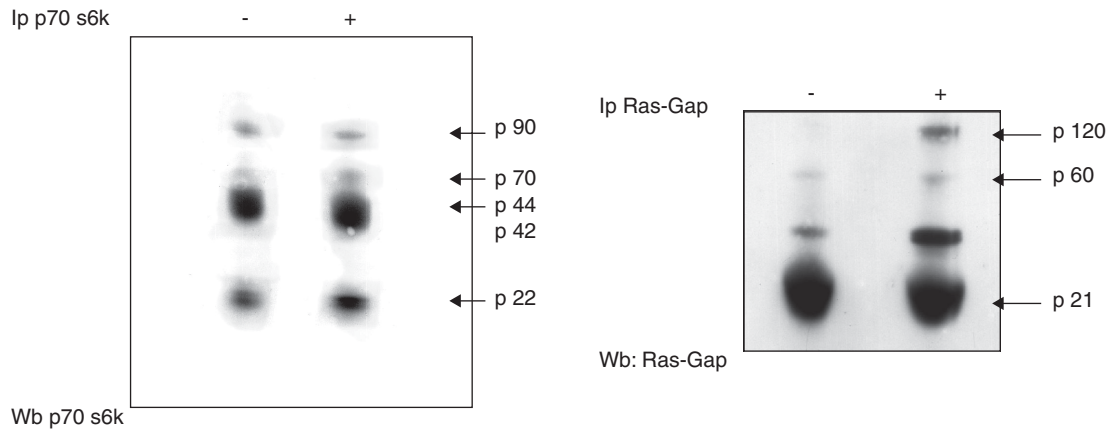


Figure 6. Right panel: immunoblot revealing a major signal of RAS and GAP with a p60/p62 associated protein (p62 src?) in *Saccharomyces boulardii*-treated rats (+) and controls (-). Left panel: immunoblot of p70 S6 kinase with associated p90 RSK, p44, p42 (ERKs?) and p21 in *S. boulardii*-treated rats (+) and controls (-).

Table I. Effects of PD098059 (inhibitor of MAPKs) on intestinal mucosal mass parameters.

	BW (g)	Intestinal length jejunum or ileum (cm)	Jejunal weight (g)	Ileal (g)	Jejunal mucosa (g)	Ileal mucosal (g)
Treated group	82.4 ± 6.0	43.16 ± 1.30	2.76 ± 0.22	2.16 ± 0.26	1.96 ± 0.44	1.47 ± 0.18
Controls	86.1 ± 6.4	41.91 ± 1.31	2.68 ± 0.05	1.89 ± 0.33	1.74 ± 0.13	1.31 ± 0.08
%	+5%	-3 %	-5.8 %	-13%	-12%	-11%
<i>p</i>	NS	NS	0.024	0.0039	0.0009	0.015

Data are means ± SD. The number of animals in each group is eight.

Abbreviations: % = differences in percentage between PD-treated group and control group; BW = body weight; NS = not significant; *p* = probability of differences between means.

Table II. Changes in jejunal disaccharidases in rats treated between days 30 and 36 with PD098059, an inhibitor of ERK_{1,2} MAPKs or with its vehicle.

Parameter	Controls (vehicle) <i>n</i> = 8	PD 098059 <i>n</i> = 8	Δ ¹	<i>p</i> ²
Lactase	19.00 ± 2.73 ³	11.36 ± 2.40	-41%	<0.001
Sucrase	135.69 ± 18.72	67.59 ± 12.01	-51%	0.0001
Maltase	706.63 ± 115 ± 00	436.06 ± 52.73	-43%	0.0014

¹Variation in percentage of PD098059-treated group vs controls.

²Probability of differences between PD098059-treated group and controls.

³Values are means ± SD; *n* = number of animals.

demonstrates that *S. boulardii* generated stimuli transduced via the pathway GRB2-SHC-CRKII-RAS-GAP-RAF-1 because each of these signaling substrates were increased in mucosal extracts of *S. boulardii*-treated rats compared to controls. This has also been documented in other cells or tissues exposed to hormonal stimuli [15,21,22]. The stimulation of this pathway ends by enhancing RAF-1 and ERK_{1,2}. This is further attested by the facts that mucosal extracts from controls and from *S. boulardii*-treated rats contained equivalent amounts of proteins (± 20 µg/ml) before immunoprecipitation and that some

key substrates such as SHC, RAF, and ERKs were tested using antibodies recognizing the diphosphorylated forms of these substrates.

Growth receptor bound 2 protein was also enhanced in *S. boulardii*-treated rats. It interacts with SHC and SOS [19], which in turn interact with CRKII. Growth receptor bound 2 protein interacts with many other tyrosine-phosphorylated proteins via its SH₂ and SH₃ domains. Interestingly, SOS-GRB2 are recruited in response to the activation of SHC, especially when residues tyr239, tyr240, and tyr317 of SHC are phosphorylated resulting in the

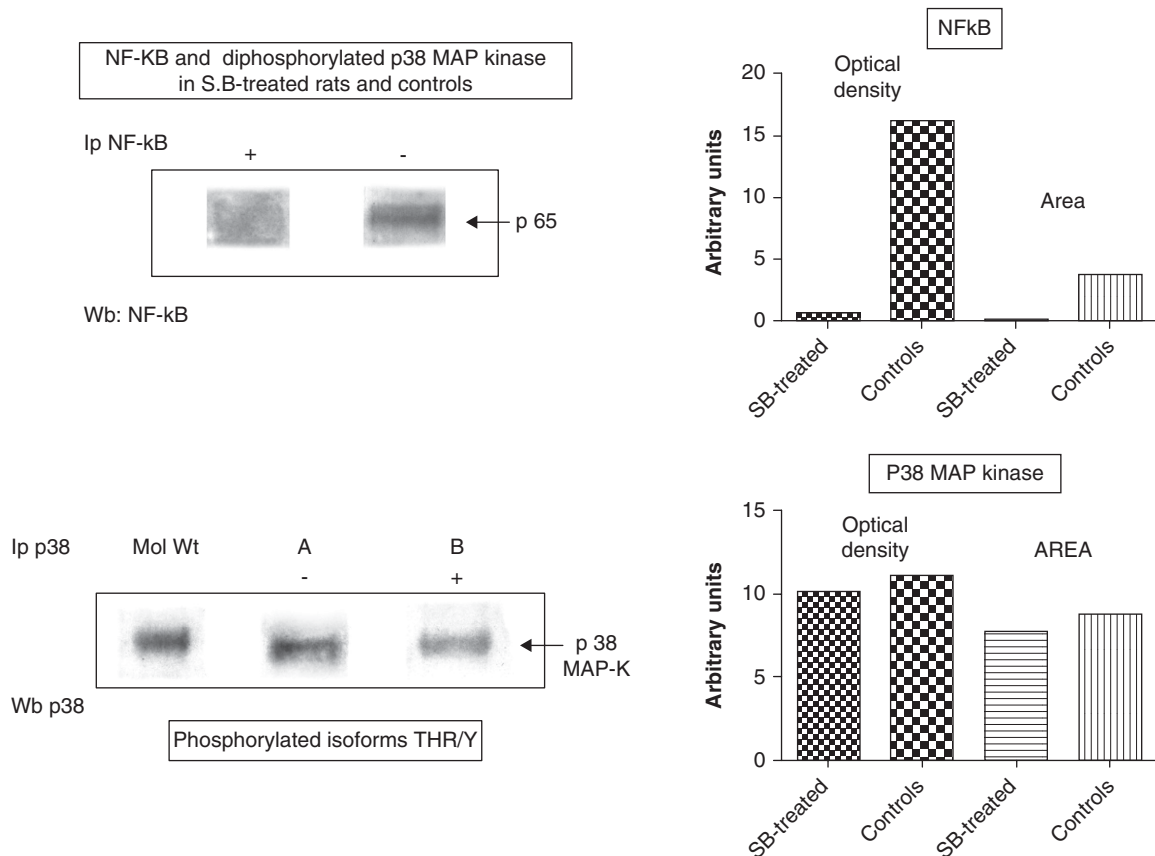


Figure 7. Upper panel left: signal of NFkB (p65 unit) detected in mucosal extracts from *Saccharomyces boulardii*-treated rats (+) and from controls (-); upper panel right: corresponding optodensitometric data giving the following results: NFkB controls $OD \times mm^2 = 16.5 \pm 0.1$ vs *S. boulardii*-treated 1.2 ± 0.1 , $n = 6$, $p < 0.01$; lower panel left: p38 MAP kinase revealed by an antibody, recognizing the double phosphorylated form (thr, tyr) of p38 in *S. boulardii*-treated rats (+) and controls (-); lower panel right: optodensitometric data: controls $OD \times mm^2 = 13 \pm 0.3$ vs *S. boulardii* 10 ± 0.2 , $n = 6$, $p < 0.05$.

stimulation of RAS-RAF-ERKs pathway [19,20]. The recruitment of GRB2 by SHC phosphorylated is well shown in Figure 4. RAF-1 or MAPKK is a serine, threonine protein kinase involved in the transduction of mitogen signals. It is an integral part of the RAS-GAP signaling pathway from the membrane to the nucleus, is the precursor of $ERK_{1,2}$, and protects cells from apoptosis by inhibiting STK_3 (serine, threonine kinase 3, a stress-activated pro-apoptotic kinase), which induces chromatin condensation and DNA fragmentation after caspase cleavage [24]. Figure 1 shows that Pi-3 kinase was more abundant in *S. boulardii*-treated rats than in controls (A+ vs B- dilution 1/20). To further confirm the effects of *S. boulardii* treatment on this key enzyme, we have tested the expression of the phosphorylated form of the regulatory unit α -p85 (Figure 3). Phosphorylated α -p85 was increased by 2.5-fold in *S. boulardii*-treated rats compared to controls. The α -p85 regulatory subunit binds to phosphorylated protein tyrosine kinases through its SH_2 domain and acts as a crucial regulator unit, mediating the binding of the p110

catalytic unit to the plasma membrane [25]. Both units have been shown to be present in the rat small intestine [15,22]. Downstream, p85 α activates PKB to regulate GLUT 4 and vesicular uptake of glucose. The α -p85 unit, when phosphorylated, interacts with LAT (linker for activation of T cells), which itself interacts through SH_2 domains with GRB2, RAP, PLC- γ_1 , SOS, and CRKII (cross-talk) [15,22,25]. The signals of SHC of CRKII were also enhanced in response of *S. boulardii* treatment.

Figure 6 (right panel) shows unequivocal evidence that the RAS-GAP pathway is stimulated by *S. boulardii*. The RAS-GAP (p120) complex is associated with a p60 ~ 62 protein, which is likely p62^{SRC}, another potential effector of p21 RAS because it contains DNA and RNA binding domains and exhibits similarities to ribonucleoproteins [26]. The cross-talk suggested here between the PI-3 kinase and the RAS-GAP-RAF pathways is an original observation published earlier by us [15,22]. The left panel of Figure 5 shows an immunoblot of immunoprecipitated extracts with an anti p70-S6 kinase antibody. Although tested with an

affinity antibody recognizing the serine, threonine phosphorylated form of the p70-S6 kinase, p70 protein was only moderately increased in abundance as were the associated proteins, p90 (p90^{RSK}), p44-42 (ERKs), and p22 (GRb2 ?).

P70-S6 kinase is a serine, threonine kinase that specifically phosphorylates ribosomal proteins in response to trophic hormones or several classes of mitogens [27]. It is mainly expressed in the small intestine. P90^{RSK} is another serine, threonine kinase acting on ribosomal proteins and the transcription factor CREB (C-AMP response element binding protein), which is implicated in the generation of circadian rhythmicity, while P90^{RSK} is activated by ERK_{1,2}. Nuclear factor kappa B are heterodimeric complexes that, once activated in the cytoplasm, migrate into the nucleus and play a role in regulation of apoptosis and of acute phase reactions [24,28]. p38MAP kinase is activated by proinflammatory cytokines and environmental stress *in vivo*. Both factors were depressed in *S. boulardii*-treated rats. The protective anti-apoptotic effects of *S. boulardii* has also been shown *in vitro*. Enterohemorrhagic *Escherichia coli* (EHEC) infection of T 84 cells in culture stimulated TNF- α synthesis and apoptosis (pro-caspases 3,8,9). *Saccharomyces boulardii* induced a decrease in TNF- α and related apoptosis in EHEC-infected cells [29]. In addition to trophic and metabolic effects on intestinal mucosa, *S. boulardii* exerts anti-inflammatory effects by inhibiting the production of several pro-inflammatory cytokines (IGF), by interfering with the global mediator of NFkB and by modulating the activity of MAPK (ERK₁, ERK₂) and p38 MAP kinase. *Saccharomyces boulardii* activates the expression of peroxysome proliferator-activated receptor-gamma (PPAR- γ) that protects from gut inflammation; the cell yeast suppresses bacterial overgrowth and host adherence by the release of a protease that cleaves *Clostridium difficile* toxin A and binds to its intestinal receptor [30]. Also, *S. boulardii* inhibits endotoxins from *E. coli* 0127B7 by dephosphorylation of the toxin due to a protein phosphatase [31].

In conclusion *S. boulardii* generates mitogen and metabolic effects that are transduced into intestinal mucosal cells by several substrates and serine, threonine and tyrosine kinases that regulate downstream the transmission of the signals from the cell apical membrane to the nucleus. The pathways involved appear to be the RAS-GAP, RAF-1, and ERK_{1,2} pathways with possible implication of GRB2, SOS, SHC, CRKII, and p85 α of Pi-3 kinase. This confirms that there is well a cross-talk between the RAS-GAP pathway and the Pi-3 kinase pathway. In addition, the probiotic decreases the signals of p38 MAP kinase and of NFkB, two initiators of pro-apoptotic transcription

factors. Further studies are necessary to find the initial factor(s) that generate from the probiotic *S. boulardii* the stimuli that activate these signaling pathways.

Acknowledgments

The authors would like to thank Bernard Hublot and Paul Bernasconi from Laboratoires Biocodex, Gentilly, France, for financial support of this research project and for helpful comments on the manuscript. Christiane Neefs is thanked for her excellent secretarial work.

Disclosure of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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