

**TRANSDUCTION PATHWAYS REGULATING THE TROPHIC EFFECTS  
OF  
*SACCHAROMYCES BOULARDII* IN RAT INTESTINAL CELLS**

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**RUNNING TITLE : TRANSDUCTION PATHWAYS OF *SACCHAROMYCES BOULARDII*  
EFFECTS**

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## ABSTRACT

*S. boulardii* is a probiotic yeast widely prescribed in a lyophilized form that determines in human and rat small intestine several effects 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 the cell. **Methods:** litters of 6 growing Wistar rats each (30 days post partum) were treated with *S.boulardii* (50µg per g body wt) or with saline during 72 hours. 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 ECL and were measured by optodensitometry. **Results:** treatment with *S.boulardii* enhanced markedly the RAS-GAP-RAF-ERK1,2 pathway with participation of GRB2, SHC, SOS, and CRKII. Unit p85a of Pi-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 which are transduced into intestinal cells downstream from the apical membrane to the nuclei, using recruitment substrates and serine, threonine, or tyrosine kinases.

**Keywords :** *Saccharomyces boulardii*, transduction pathways, mucosal disaccharidases, D-glucose uptake, polyamines, probiotic

*Saccharomyces boulardii* (*S. 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 vesicles with a corresponding increase in the sodium D-glucose co-transporter 1 (SGLT-1) [11]. These effects are, at least in part, 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 in the intestinal lumen enzymes like sucrase [7],  $\alpha,\alpha$ -trehalase [9], leucine-aminopeptidase [8] and a novel protein phosphatase that inhibits *Escherichia coli* O<sub>55</sub>B<sub>5</sub> endotoxin by dephosphorylation [14].

The objectives of the present study were to determine whether the trophic effects generated by *S. boulardii* could be regulated in rat intestinal mucosal extracts by known signalling substrates and phosphorylated kinases.

## MATERIALS AND METHODS

**Media and culture conditions.** *S. boulardii* cells were inoculated in YPD (yeast extract, 0.5%; peptone, 2%; glucose, 2%; DIFCO, Detroit, MI, U.S.A.) media and were grown at 30°C with moderate shaking [ 7 ]. *S. boulardii* was prepared in a lyophilized form (100 mg per flask, biologic activity  $2.9 \times 10^9$  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 per group were studied between day 30 and 34 postpartum corresponding to the post weaning period of growth. *S. boulardii* was administered to rats (n=10) (100 mg per flask, biologic activity  $2.9 \times 10^9$  viable cells/ml ) at the dose of 50 µg of lyophilized yeast cells per gram body weight per day , in 0.1ml saline by nasogastric intubation, twice daily [7] . Control groups (n=10) were treated according to the same schedule and received equal volumes of saline. PD 098059, an inhibitor of MAP kinase kinase (MAPKK ) and of ERK<sub>1,2</sub> , was purchased from Santa Cruz Biotech (CA,USA). The inhibitor was administered intraperitoneally , twice daily [ 15 ] at the dose of 2 µg per g body weight per day from day 30 to day 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 ileocaecal 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<sup>®</sup> paper and frozen in liquid nitrogen ( -170° ) until use.

**Enzymes assays.** Disaccharidases activities were measured on jejunal mucosal samples using the micromethod of Messer and Dahlqvist [ 16 ] . One unit equals 1  $\mu$ mole of glucose formed per minute and per gram protein. Protein content was determined by the method of 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 in each sample 100 mg protein ;It was thereafter diluted 5 fold (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 $\mu$ l) including pepstatin, leupeptine and PMSF. 100 $\mu$ l of protein A Sepharose 4B, diluted in RIPA buffer (1/1) was added to each sample and mixed by rotation for 4h at 4°C. Thereafter, the samples were centrifuged at 24.000 g for 5min at 4°C. The pre-cleared supernates were collected and mixed with 10  $\mu$ l IgG purified antibodies by rotation overnight at 4°C. 100  $\mu$ l of protein A sepharose 4B was then added to the samples and mixed by rotation for 4h at 4°C. The sepharose beads were washed twice with RIPA buffer and once with TRIS, 10 mM buffer. After the last wash, 5  $\mu$ l bromophenol blue (Invitrogen, Carsbas. CA., USA) diluted in 10  $\mu$ l 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, N.J. USA). This novel procedure allows the detection of nanograms of antigen by chemoluminescence (ECL) without showing co-immunoprecipitation of the heavy and light chains of the IgG antibody.

**Antibodies.** To assess whether *S. boulardii* could activate the rat integrin receptor , a peptide of 37 aminoacids -<sup>1</sup>NFLAKLNENHSGELWKGRWQGNDIVVKVLKVRDNSTR<sup>37</sup> corresponding to a highly conserved sequence of rat integrin receptor was synthesized .This peptide (AA 194 – 230) matched similar peptides from mouse (identities 35/37, 94%), human (identities 34/37, 91%) and chicken (identities 28/37, 75%). Rabbits were immunized with the above peptide. A polyclonal antibody was

generated and IgG fractions were purified . Unless otherwise indicated, all other antibodies used were polyclonal rabbit antibodies purchased from Santa Cruz Biotech. (Santa Cruz, CA., USA). Each antibody recognized an intestinal transduction substrate or a tyrosine – serine- or - threonine kinase demonstrated by previous studies to be present in small bowel mucosal extracts ( 15 ). GRB2 is an adapter molecule that provides a critical link between cell surface growth factor receptors and the RAS signalling pathway. The antibody used was affinity purified and raised against a peptide (C-23) mapping the C-terminus of GRB2 of rat origin (p 21). The antibody used for 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 (thre 421 ,ser 424) of the C-terminus peptide of rat p70S6 kinase. Extracellular signal regulated kinase 1 and 2 (ERK<sub>1, 2</sub> ) were tested by antibodies raised against the non phosphorylated form of ERK<sub>1, 2</sub> and the double phosphorylated form, i.e. a short amino sequence containing phosphorylated thre 202 and tyr 204 for ERK<sub>1</sub> AND thre 185 and tyr 187 for ERK<sub>2</sub> of human origin. The protein serine, threonine kinase (RAF-1) is a MAPKK which was assessed by a purified antibody raised against a short amino acid sequence containing the double phosphorylated form ( thre 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 alpha, p85 beta, p55 and p50 units of the complex enzyme. To clearly demonstrate the effect of *S. boulardii* on Pi-3 kinase we used also an affinity purified antibody raised against a short amino acid sequence containing the phosphorylated form ( tyr-508) of  $\alpha$  p85 regulatory unit of human origin. p38 MAP kinase was tested using an affinity purified antibody raised against a phosphopeptide including isoforms p38 beta, gamma and delta , with sequences

surrounding thre-183 , tyr- 185 of p38 gamma, thre -180 , tyr-182 of p38 beta, and thre-180 , tyr-182 of p38 delta. The transcriptional motif (p65) of heterodimeric NF $\kappa$ B was studied in controls and *S. boulardii* - treated rats using a polyclonal antibody raised against a polypeptide (aa 1 – 286) of NF $\kappa$ B of human origin which contains the transcriptional activator motif (p65). The other transduction factors studied were the beta 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 du- or tri- plicate. The intensity and amount of signals were measured by optodensitometry , giving arbitrary units of optical density with adjusted volume : O.D x mm<sup>2</sup> and area All optodensitometric measurements were made more than 4 times on each Western blot All data are means  $\pm$  SD. SD less than 10% of the mean, are not depicted . Mucosal mass parameters and enzyme activities from treated groups and controls were tested for statistical significance (p<0.05) with ANOVA (F test) followed by unpaired Student' t test if allowed by the F value. Where not appropriate, differences between means were tested using the non parametric Mann Withney “U “ test; Differences were considered significant for p<0.05.

## 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<sup>+</sup> at 1/20 and 1/40 dilutions) and controls ( B- at 1/20 and 1/40 dilutions ) . Figure 1 identifies 4 units : the catalytic unit p110 (weak signal), the two regulating units p85<sub>α</sub> and p85<sub>β</sub> isoforms, and p55/p50 proteins which appear in one band and have previously been evidenced in rat brain, muscle, liver and intestine [ 18 ]. As shown in Figure 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<sup>+</sup> ,dilution 1/20, and in the corresponding optodensitometric data shown in figure 2 for p85<sub>α</sub>, p85<sub>β</sub> and p50/55. The p110 signal was too weak for interpretation. These units co-immunoprecipitated because our antibody was raised against the entire NH<sub>2</sub> – SH<sub>2</sub> domain of Pi-3 kinase which is common to p85<sub>α</sub>, p85<sub>β</sub>, p55 and p50 [ 18 ]. To further confirm that the signal of *S. boulardii* is regulated by p85<sub>α</sub> , we used an antibody recognizing the tyrosine phosphorylated form (tyr 508) of p85<sub>α</sub> . Figure 3 demonstrates that the signal of the p85<sub>α</sub> phosphorylated form was much more intense in *S. boulardii* -treated rat (ODxmm<sup>2</sup> : 27.75 ± 1.1 p<0.02 ; area : 24.13 ± 0.9 p<0.05 vs controls ) than in controls (ODxmm<sup>2</sup> : 14.09 ± 0.9; area : 14,1 ± 0,5 ).

GRB2 ( p21 ) was detected in both groups as a strong signal (Figure 1) but was only slightly increased (OD x mm<sup>2</sup> : 18,57 ± 1,3 vs 16,71 ± 1,1 p=ns) (area = 16,99 ± 0,7 vs 15,70 ± 0,2 p=ns) in the group treated with the probiotic. GRB2 is a small cytoplasmic protein containing two SH<sub>3</sub> domains and one SH<sub>2</sub> domain . GRB2 interacts in response to extra- membrane stimuli and binds to mammalian SOS (mSOS not shown here) and SHC via its SH<sub>2</sub> domain . Isoforms ( p52, p46 ) of SHC ,when phosphorylated , function as initiators of the RAS signalling cascade after their interaction with the complex GRB2-SOS . As shown in figure 4 ,we found 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 likely GRB2 .In addition, the complex SOS and GRB2 interacts with CRKII another adapter protein



that bind to GRB2 through 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.(  $p < 0.01$  vs controls)

Like as in target cell lines stimulated in vitro with growth factors, we have detected in intestinal mucosal extracts from weaned rats (day 36) a 120 kDa protein identified as GAP (the mean GTPase – activating protein of the normal form of p21 RAS) (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 aminoacids 171-443 which include 2 SH<sub>2</sub> domains and 1 SH<sub>3</sub> domain) revealed a major signal of ~ 120 kDa ( GAP) (OD x mm<sup>2</sup> :  $9.47 \pm 0.12$ ; area :  $8.77 \pm 1.08$  no controls ) a weak signal at ~ 60 kDa (likely p62<sup>SRC</sup>) (OD x mm<sup>2</sup>  $5.95 \pm 0.7$ ; area :  $6.64 \pm 0.9$   $p = 0.05$ ), a marked signal of 44 kDa (likely ERK<sub>1,2</sub>) (OD x mm<sup>2</sup> :  $11.9 \pm 1.2$ ;  $p < 0.05$  vs controls; area :  $14.82 \pm 0.16$   $p < 0.002$  vs controls) 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 x mm<sup>2</sup> :  $2.27 \pm 0.3$ ; area:  $2.63 \pm 0.86$   $p = ns$ ) and p44 decreased (OD x mm<sup>2</sup> :  $3.22 \pm 0.8$ ; area:  $3.04 \pm 1.06$   $p < 0.05$  vs controls) 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 x mm<sup>2</sup> : 57.56 vs 53.71 in controls ;not significant ) 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 have identified the phosphorylated form (thre 598, ser 601) of RAF-1 (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 x mm<sup>2</sup>:  $50 \pm 0.2$   $p < 0.01$ ; area :  $32 \pm 0.1$   $p < 0.02$  ) compared to controls (OD x mm<sup>2</sup>:  $37 \pm 0.1$ ; area :  $22 \pm 0.6$ ) (Figures 1 ) . ERK<sub>1</sub> and ERK<sub>2</sub> ( extracellular signal – activated kinases 1 and 2) are mitogenic activated protein kinases (MAPK) 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. ERKs can be directly activated on cell surface by endoluminal stimuli and once phosphorylated, migrate into the nucleus. Their precursor is RAF-1. As shown in Figures 1 (upper right panel) the signal of ERK1 was enhanced by 3 times in *S.boulardii*-treated rats (OD x mm<sup>2</sup> : 15 ± 0.2 p<0.01 vs controls; area : 17 ± 6.2 p<0.02) compared to controls (OD x mm<sup>2</sup> : 5.8 ± 0.09; area : 5.7 ± 0.08). To further demonstrate that the trophic signal(s) of *S. boulardii* are regulated by ERKs we used an antibody recognizing the diphosphorylated forms of ERK<sub>1</sub> and of ERK<sub>2</sub>. As shown in Figure 3 (left panel), ERK<sub>1</sub> and ERK<sub>2</sub> were detected as a large aggregated complex which was more marked in *S. boulardii*-treated rats (OD x mm<sup>2</sup> : 41.5 ± 0.5, p<0.01; area : 39,6 ± 0.22, p<0.02) than in controls (OD x mm<sup>2</sup> : 28.3 ± 0.3; area : 23.4 ± 0.14). The signals were fused because molecular weights of ERK<sub>1</sub> and ERK<sub>2</sub> are very close and there is more than 90% homology between the structure of the two serine-threonine kinases. To further provide evidence that the ERK<sub>1</sub> / ERK<sub>2</sub> pathway mediate the trophic signals generated by *S. boulardii*, we treated weaned rats (day 30 to day 36 post partum) interperitoneally with PD-098059 a specific inhibitor of MAPKK and in turn of ERK<sub>1</sub> and ERK<sub>2</sub>. The inhibitor was administrated by intraperitoneal injection at a low dose (2µg /g body weight twice daily) (median of lethal dose = 200 µg/g) for 4 days, 1 hour before the administration of *S. boulardii*. Control rats received the vehicle of PD0980059. 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 1, mucosal mass, whether jejunal mucosa or ileal mucosa, was significantly (p< 0.015 n = 16) lower by -11 to -13% in the experimental group compared to the control group. Changes in the specific activity of disaccharidases are detailed in Table 2. 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 ERK<sub>1</sub> or ERK<sub>2</sub>. 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<sup>RSK</sup> and p70S6kinase were associated together and with other proteins including p44/p42 and p22. The former are likely ERK<sub>1</sub> and ERK<sub>2</sub>. 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 NFκB and of p38 MAPkinase and on the right side the corresponding optodensitometric data. NFκB ( 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 x mm<sup>2</sup> .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 mucosal extracts of growing rats in previous studies (15) and in the present study using our sensitive method of immunoprecipitation and immunoblotting on mucosal extracts from both treated and control rats, that were equivalent in terms of age, weight, treatment schedules, nutrition and protein concentration. Results concerned at least epithelial cells because in a preliminary experiment on isolated enterocytes similar differences between ERK-1 and ERK-2 were observed.( data not shown). However, because we used whole mucosal extracts as previously (15) we cannot exclude the inclusion of other cell types such as lymphocytes, fibroblasts, macrophages etc... First of all, the non phosphorylated form (Figure 1) and the diphosphorylated forms of ERK-1 (p44) and ERK-2(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 PD-098059 to *S. boulardii*- treated rats at low dosis (2µg/g), clearly inhibited after 96 hours both mucosal mass (-11 to -13% compared to controls)(Table 1) and the expression of BBM disaccharidases (-41 to -51% vs controls) (Table 2) [15]. ERK<sub>1,2</sub> (p44,p42) are extracellular signal regulated kinases activated by extracellular stimuli which migrate downstream into the cytoplasm and nuclei [19] where they are involved in both initiation and regulation of meiosis, mitosis and postmitotic functions of differentiated cells by

phosphorylating a number of nuclear transcription factors mainly ELK-1 (ETS family, transcription activators that play a role in regulating epithelial cell differentiation and proliferation) [20] and EIF4EBP1 (eukaryotic translation initiation factor 4E-B binding protein-1, required for initiation of translation). On the other hand, our study demonstrates that *S. boulardii* generated stimuli transduced via the pathway GRB2-SHC-CRKII-RAS-GAP-RAF-1 because each of these signalling substrates were increased in mucosal extracts of *S. boulardii*-treated rats compared to controls. This has been also documented in other cells or tissues exposed to hormonal stimuli [ 21 ] The stimulation of this pathway ends by enhancing RAF-1 and ERK<sub>1,2</sub> .. THIS is further attested by the facts that mucosal extracts from controls and from *S. boulardii* – treated rats contained equivalent amounts of proteins ( $\pm 20 \mu\text{g/ml}$ ) before immunoprecipitation and that some key substrates such as SHC, RAF and ERKs were tested using antibodies recognizing the diphosphorylated forms at these substrates.

GRB2 was also enhanced in *S. boulardii* - treated rats. GRB2 interacts with SHC and SOS [ 22 ] which in turn interacts with CRKII . GRB2 interacts with many other tyrosine-phosphorylated proteins via its SH<sub>2</sub> and SH<sub>3</sub> 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 stimulation of RAS-RAF-ERKs pathway [ 23 ]. 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 integrate part of the RAS-GAP signalling pathway from the membrane to the nucleus, is the precursor of ERK<sub>1,2</sub> and protects cells from apoptosis by inhibiting STK<sub>3</sub> (serine, threonine kinase 3, a stress-activated pro-apoptotic kinase, which induce 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- dil. 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  $\alpha$  p85 (Figure 3 ). Phosphorylated  $\alpha$  p85 was increased by 2.5 fold in *S. boulardii* - treated rats compared to controls. The  $\alpha$  p85 regulatory subunit binds to phosphorylated protein tyrosine kinases through its SH<sub>2</sub> 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 ]. Downstream, p85a activates PKB to regulate GLUT 4 and vesicular uptake of glucose. The  $\alpha$  p85 unit, when phosphorylated interacts with LAT (Linker for activation of T cells) which itself interacts through SH<sub>2</sub> domains with GRB2, RAP, PLC-gamma<sub>1</sub>, SOS and CRKII. ( cross-talk)[15,26] .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<sup>SRC</sup> another potential effector of p21 RAS because it contains DNA and RNA binding domains and exhibits similarities to ribonucleoproteins [ 27 ].The cross-talk suggested here between the PI-3 kinase and the RAS-GAP-RAF pathways is an original observation published earlier by us [ 15,26 ] . 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<sup>RSK</sup> ), p44-42 (ERK<sub>s</sub>) and p22 (GRb2 ?).

P70-S6 kinase is a serine, threonine kinase which phosphorylates specifically ribosomal proteins in response to trophic hormones or several classes of mitogens.[ 28 ] It is mainly expressed in the small intestine. P90<sup>RSK</sup> is another serine, threonine kinase, acting on ribosomal proteins and the transcription factor CREB (C-AMP response element binding protein). CREB is implicated in the generation of circadian rhythmicity, while P90<sup>RSK</sup> is activated by ERK<sub>1, 2</sub>. NFkB are heterodimeric complexes which ,once activated in the cytoplasm, migrate into the nucleus and play a role in

regulation of apoptosis and of acute phase reactions [ 29 ]. 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 T84 cells in culture stimulated TNF-alpha synthesis and apoptosis (pro-caspases 3,8,9 ) . *S.boulardii* induced a decrease in TNF-alpha and related apoptosis in EHEC-infected cells [ 30 ].

**In conclusion:** Our study demonstrates that oral administration of *Saccharomyces boulardii* generates mitogen and metabolic stimuli that are transduced into intestinal mucosal samples by several substrates including serine, threonine and tyrosine kinases that regulate downstream the transmission of the signals from the apical membrane to the nucleus; The pathways involved appear to be the RAS-GAP,RAF-1 and ERK1;2 pathway with possible implication of GRB2,SOS,SHC,CRK II and p85a 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 p38MAP kinase and of NFkB, two initiators of proapoptotic transcription factors .Further studies are necessary to find the initial factor(s) that generate from the probiotic *S. boulardii* the trigger(s) wich activate these signalling pathways.

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

## ABBREVIATIONS

**BBM** : Brush border membrane

**GRB2** : Growth receptor bound 2 protein

**RAS ( p21 )** : binds GDP/GTP and has intrinsic GTPase activity

**GAP** : GTPase stimulating normal p21 RAS

**SOS** : Son of sevenless

**ERK** : Extracellular signal regulated kinase

**RAF-1** : ( MAPKK ) Mitogen activated protein kinase kinase

**Pi-3 k** : Phosphatidylinositol –3 kinase

**PKB** : Protein kinase B

**PKC**: Protein kinase C

**CRK II** : proto-oncogen C-crk II

**SGLT-1** : Sodium-D-glucose cotransporter – 1

**MAPKK** : Mitogen activated kinase kinase

**P38 MAPK** : p38 Mitogen activated kinase

**PMSF** : phenylmethanesulphonylfluoride

**NFkB** : ( p65 unit ) Nuclear Factor Kappa B , transcription factor p65

**IgG**: Immunoglobulin G

**Elk-1**: ETS domain containing protein ELK-1 ( transcription factor)

**STK3** : stress-activated pro-apoptotic kinase

**SHC** : Sarc homology 2 domain containing-transforming protein C1



## TITLES AND LEGENDS TO FIGURES

**Figure 1** . Four immunoblots revealing changes in Pi-3 kinase ( upper left ), ERK1 ( upper right ), GRB2 ( lower left ) and diphosphorylated RAF ( on threonine 598, serine601)( lower right ) in *S. 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 .

**Figure 2** . Optodensitometric data ( OD in arbitrary units adj. vol.x mm<sup>2</sup> and area) measured for the subunits p85a , p85b , p55 /p50 (lower half of the surface area of the signal) in *S.boulardii* – treated rats and controls .

**Figure 3.** Upper graphs : ( right) immunoblot of the phosphorylated form of p85a (on threonine 508) in mucosal extracts from *S. boulardii*- treated rats ( + ) and controls ( - )( left ): corresponding optodensitometric data ; lower graphs ( right ) immunoblot of the diphosphorylated forms of ERK1 and ERK2 (see material and methods ) in *S. boulardii* treated-rats ( + ) and controls ( - ) . ( left ): corresponding optodensitometric data

**Figure 4** . On the left : immunoblot showing SHC ( p52,p46), p66 was not detected and p21 (likely GRB2) in *S.boulardii*-treated rats ( + ) and in controls ( - ) ; on the right : corresponding optodensitometric data

**Figure 5** . On the left : immunoblot showing CRKII in *S. boularidi*-treated rats ( + ) and controls ( - ); on the right : corresponding optodensitometric data.

**Figure 6 .** Right pannel : immunoblot revealing a major signal of RAS and GAP with a p60/p62 associated protein ( p62 src ?) in *S. 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 ( - ).

**Figure 7 .** Upper pannel left : signal of NFkB ( p65 unit ) detected in mucosal extracts from *S. boulardii*-treated rats ( + ) and from controls ( - ) ; upper panel right : corresponding opto-densitometric data ; lower pannel left : p38 MAPkinase revealed by an antibody, recoginzing the double phosphorylated form (thr., tyr.) of p38 in *S. boulardii*-treated rats ( + ) and controls ( - ) ; lower panel right : corresponding optodensitometric data.

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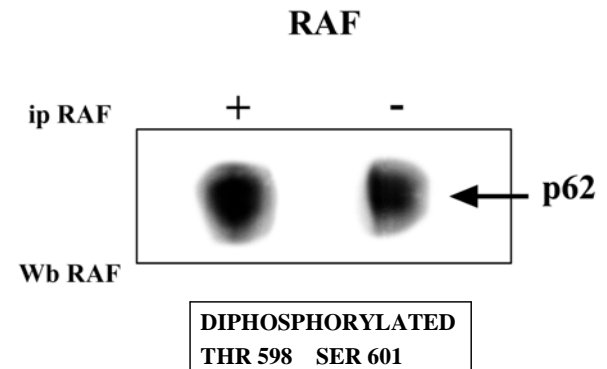
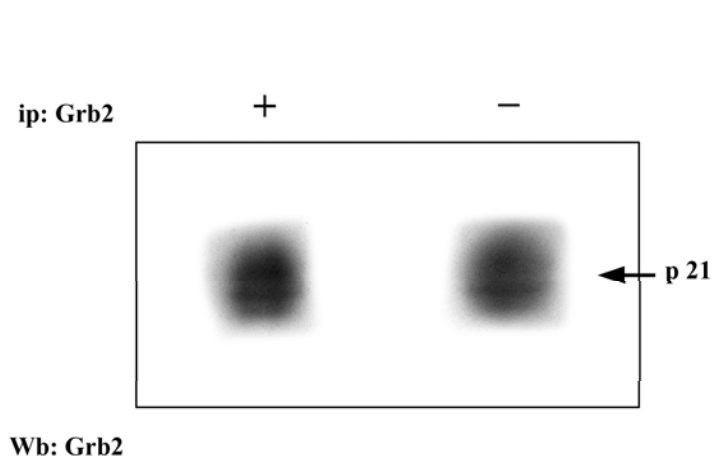
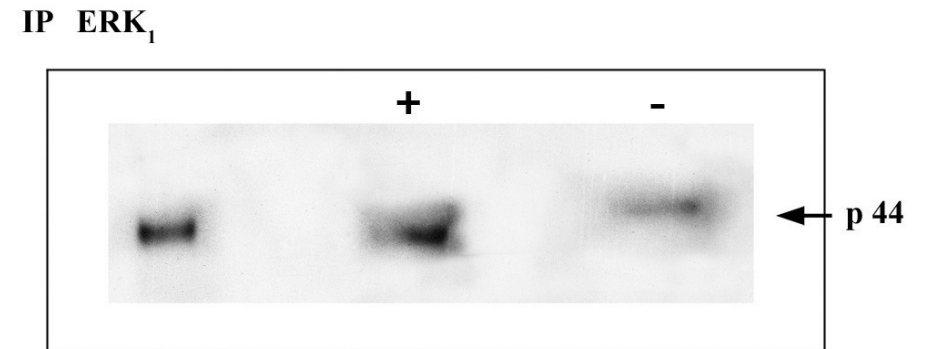
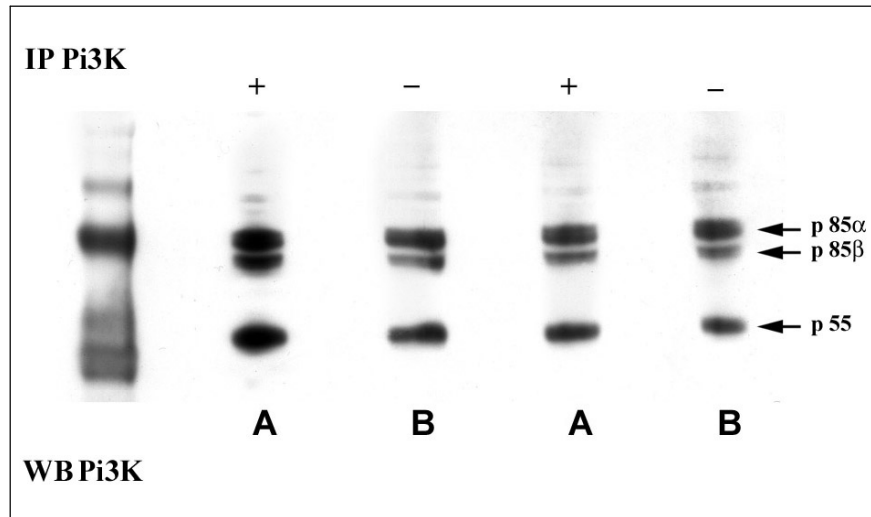




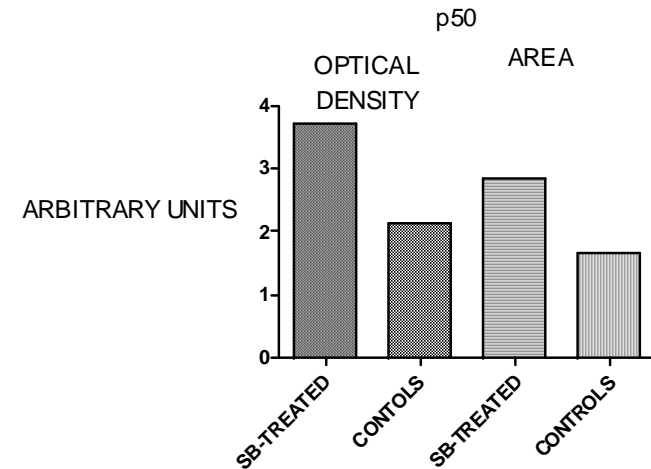
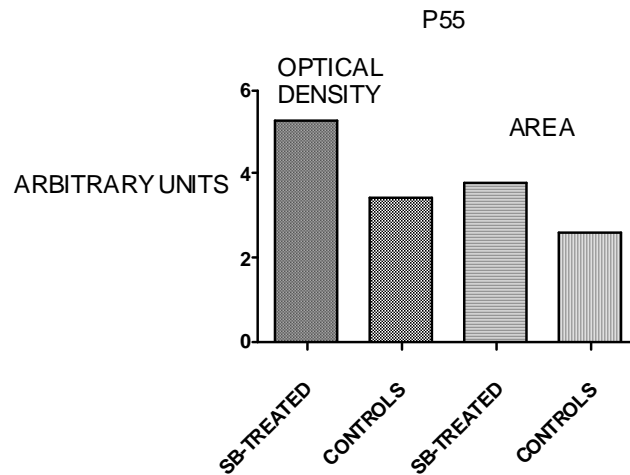
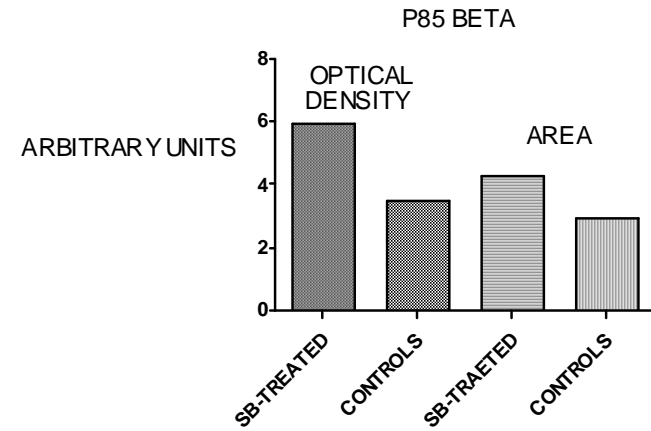
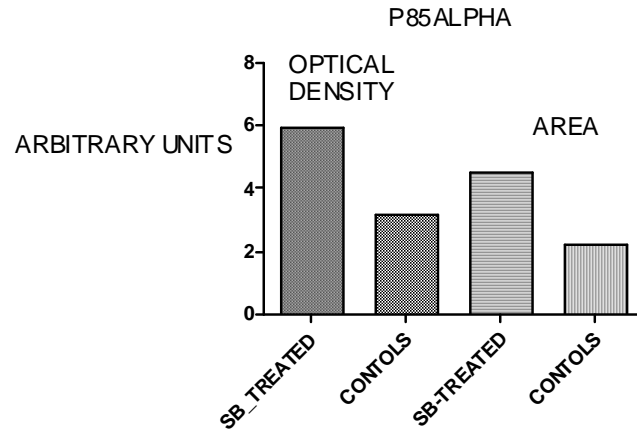




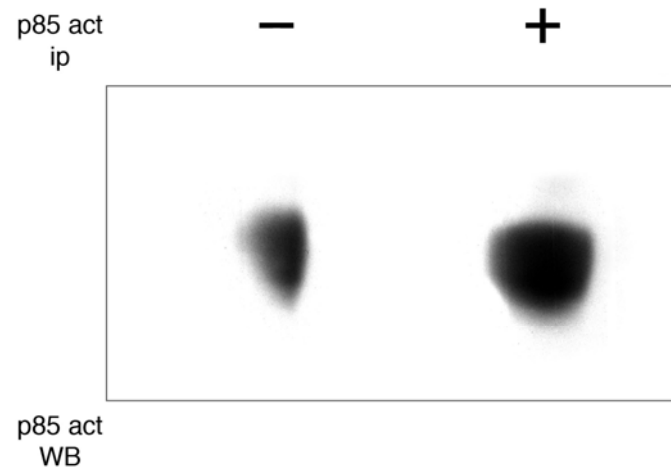
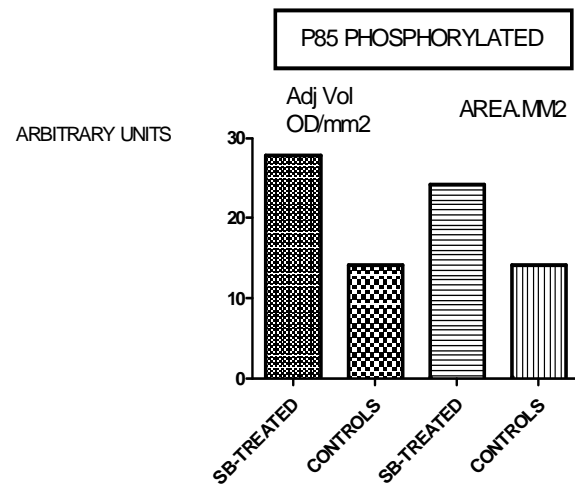
# CHANGES IN PI-3K, ERK1, Grb2 AND RAF IN RESPONSE TO *S. boulardii*



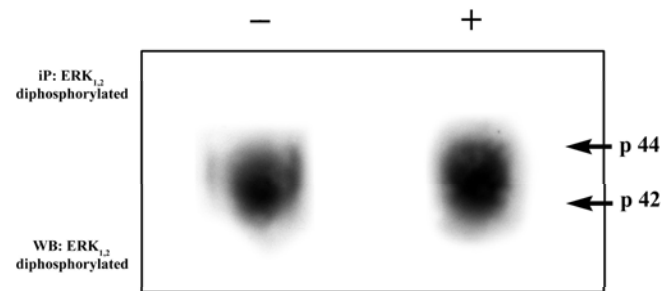
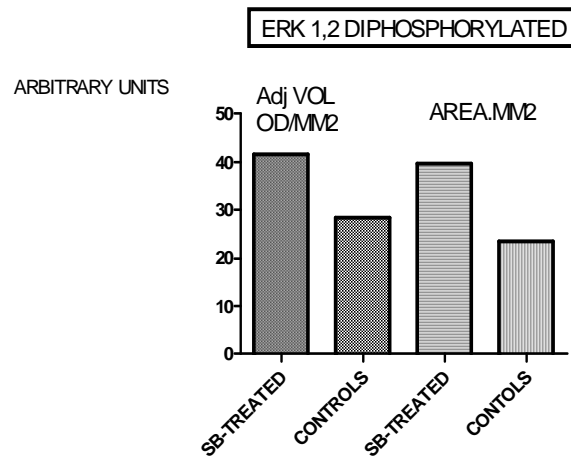
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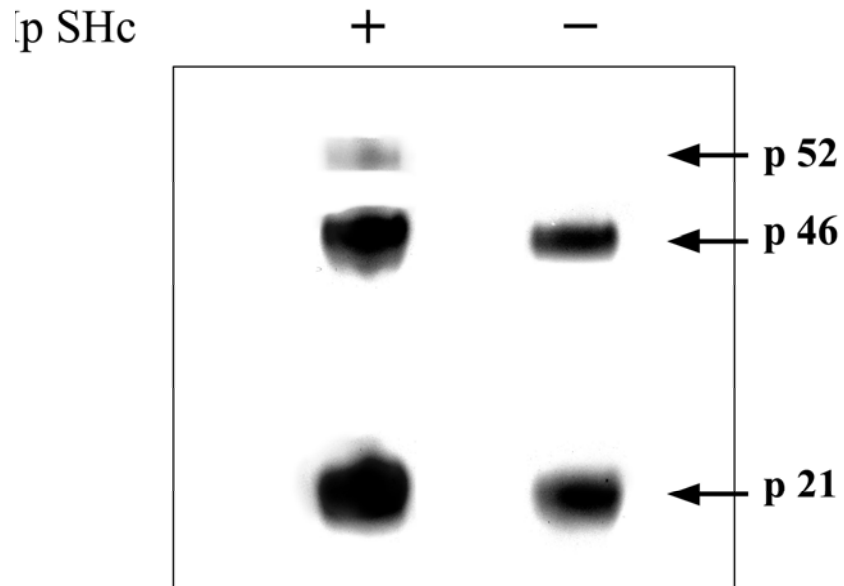
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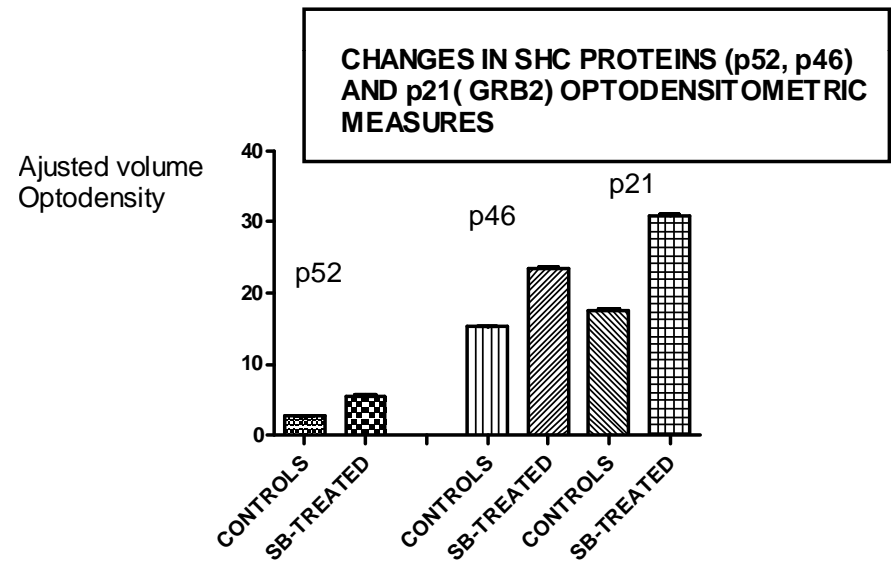
## ERK1,2 diphosphorylated



# SHC PROTEINS IN *SB*-TREATED RATS AND CONTROLS



Wb SHc

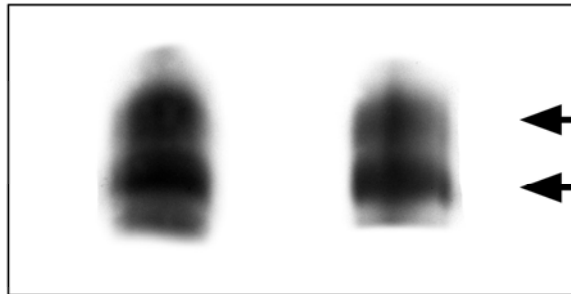


## CHANGES IN CRKII IN RESPONSE TO *S. BOULARDII*

ip CRK II

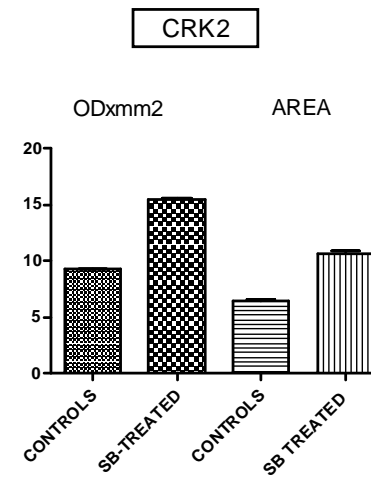
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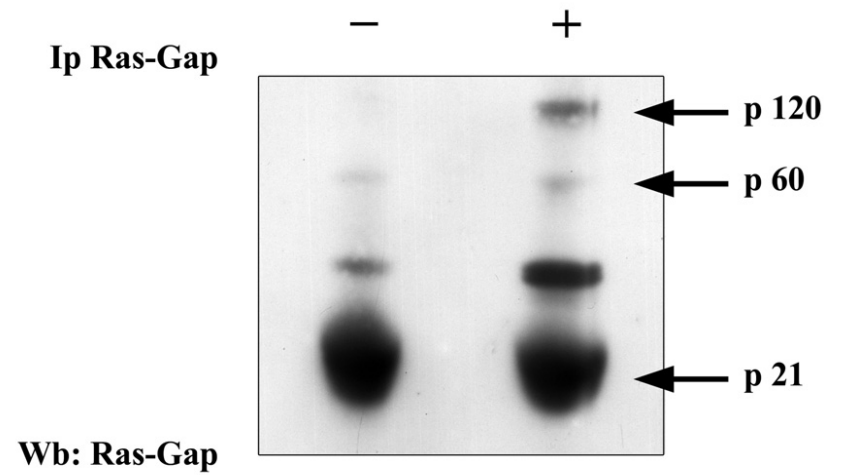
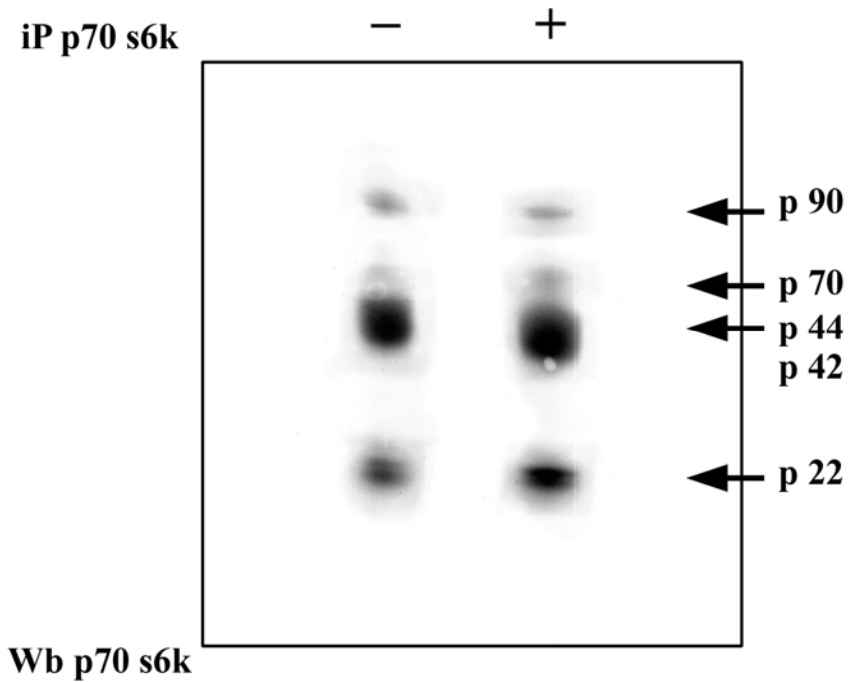


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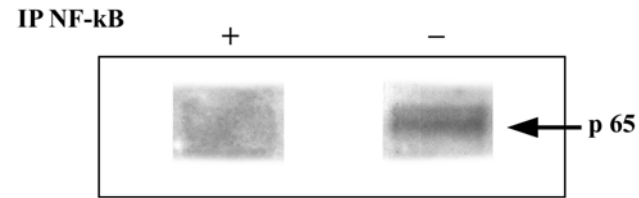


# CHANGES IN p70p90 S6 KINASE, AND Ras-Gap IN RESPONSE TO *S.boulardii*

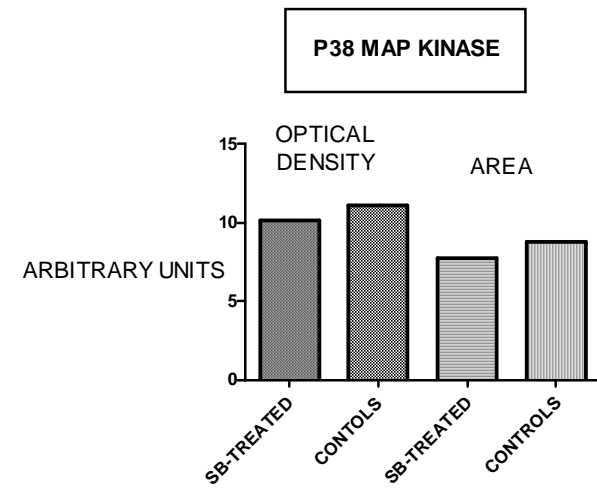
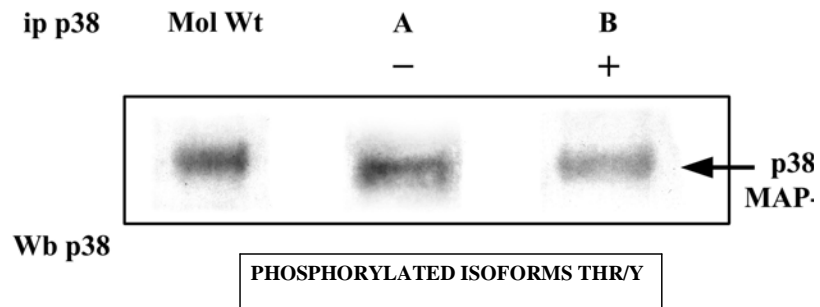
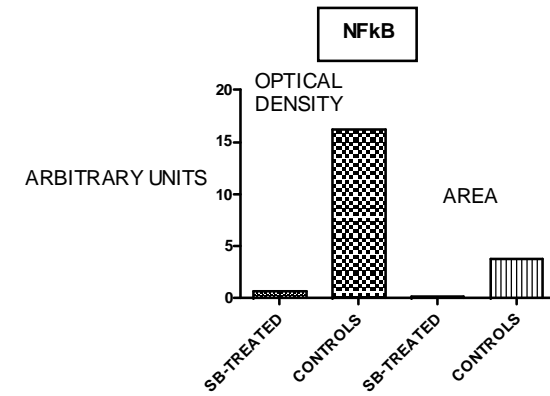




# **NF-KB AND DIPHOSPHORYLATED p38 MAP KINASE IN S.B-TREATED RATS AND CONTROLS**



**Wb: NF-kB**



**Table 1. Effects of PD 098059 (inhibitor of MAP kinases )**  
**on Intestinal Mucosal Mass Parameters**

	<b>BW (g)</b>	<b>Intestinal Length Jejunum or ileum (cm)</b>	<b>Jejunal WT (g)</b>	<b>Ileal (g)</b>	<b>Jejunal mucosa (g)</b>	<b>Ileal mucosal (g)</b>
<b>CONTROL GROUP</b>	<b>82,4 ± 6.0</b>	<b>43.16 ± 1.30</b>	<b>2,76 ± 0,22</b>	<b>2,16 ± 0,26</b>	<b>1,96 ± 0,44</b>	<b>1,47 ± 0,18</b>
<b>PD – TREATED</b>	<b>86,1 ± 6,4</b>	<b>41,91 ± 1,31</b>	<b>2,68 ± 0.05</b>	<b>1,89 ± 0.33</b>	<b>1,74 ± 0.13</b>	<b>1,31 ± 0.08</b>
	<b>+ 5%</b>	<b>- 3 %</b>	<b>- 5,8 %</b>	<b>- 13 %</b>	<b>- 12 %</b>	<b>- 11 %</b>
<b>P<sup>i</sup></b>	<b>ns</b>	<b>ns</b>	<b>0.024</b>	<b>0.0039</b>	<b>0.0009</b>	<b>0.015</b>

<sup>i</sup> P : probability of differences between means.. % differences in per cent between PD-TREATED GROUP AND CONTROL GROUP ; ns = not significant ; data are means +/- SD . The number of animals in each group is eight

**Table 2 . Changes in jejunal disaccharidases in rats treated between day 30 and day 36 with PD098059, an inhibitor of ERK<sub>1,2</sub> MAP Kinases or with its vehicle.**

<b>PARAMETER</b>	<b>Controls (vehicle) N = 8</b>	<b>PD 098059 N = 8</b>	<b><math>\Delta^1</math></b>	<b>P</b>
<b>Lactase</b>	<b>19.00 <math>\pm</math> 2.73 <sup>2</sup></b>	<b>11.36 <math>\pm</math> 2.40</b>	<b>- 41%</b>	<b>&lt; 0.001<sup>3</sup></b>
<b>Sucrase</b>	<b>135.69 <math>\pm</math> 18.72</b>	<b>67.59 <math>\pm</math> 12.01</b>	<b>- 51 %</b>	<b>= 0.0001</b>
<b>Maltase</b>	<b>706.63 <math>\pm</math> 115 <math>\pm</math> 00</b>	<b>436.06 <math>\pm</math> 52.73</b>	<b>- 43 %</b>	<b>= 0.0014</b>

<sup>1</sup>  $\Delta$  Variation in percent of PD098059 – treated group vs controls.

<sup>2</sup> Values are means  $\pm$  SD; n = number of animals

<sup>3</sup> P : probability of differences between PD 098059 – treated – group and controls