**Saccharomyces boulardii** Inhibits EGF Receptor Signaling and Intestinal Tumor Growth in Apem Min Mice

**BACKGROUND & AIMS:** *Saccharomyces boulardii* (Sb) is a probiotic yeast with anti-inflammatory and antimicrobial activities and has been used for decades in the prevention and treatment of a variety of human gastrointestinal disorders. We reported previously that Sb modulates host inflammatory responses through down-regulation of extracellular signal-regulated kinase (Erk)1/2 activities both in vitro and in vivo. The aim of this study was to identify upstream mediators responsible for extracellular signal-regulated kinase (Erk)1/2 inactivation and to examine the effects of Sb on tumor development in Apem mice. **METHODS:** Signaling studies of colon cancer cells were done by western blot. Cell proliferation was measured by MTS and BrdU assay. Apoptosis was examined by flow cytometry, tunel assay and caspase assay. Apem mice were orally given Sb for 9 weeks before sacrifice for tumor analysis. **RESULTS:** We found that the epidermal growth factor receptor (EGFR) was deactivated upon exposure to Sb, leading to inactivation of both the EGFR-Erk and EGFR-Akt pathways. In human colonic cancer cells, Sb prevented EGF-induced proliferation, reduced cell colony formation, and promoted apoptosis. HER-2, HER-3, and insulin-like growth factor-1 receptor were also found to be inactivated by Sb. Oral intake of Sb reduced intestinal tumor growth and dysplasia in C57BL/6J Min/+ (Apem) mice. **CONCLUSIONS:** Thus, in addition to its anti-inflammatory effects, Sb inhibits EGFR and other receptor tyrosine kinase signaling and thereby may also serve a novel therapeutic or prophylactic role in intestinal neoplasia.

**Materials and Methods**

**Cells and Reagent**

HT29, SW480, and HCT-116 cells were obtained from American Type Culture Collection. HT29 and SW480 cells were cultured in Dulbecco’s modified Eagle medium (DMEM), whereas HCT-116 were cultured in McCoy’s 5A modified media, supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mmol/L L-glutamine (GIBCO, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO2 incubator at 37°C. Recombinant human (rh) epidermal growth factor (EGF) and recombinant human Neuregulin (NRG-1) were pur-

**Abbreviations used in this paper:** EGF, epidermal growth factor receptor; Erk, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor-1 receptor; MAP, mitogen-activated protein; PCNA, proliferating cell nuclear antigen; PKC, protein kinase C; Sb, Saccharomyces boulardii; SbS, Sb culture supernatant.

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chased from R&D Systems (Minneapolis, MN). Antibodies against Erk, MEK, CamKII kinases, protein kinase C (PKC), EGFR, insulin-like growth factor-1 receptor (IGF-1R), HER-2, HER-3, and Akt, phosphorylated and/or nonphosphorylated forms, were purchased from Cell Signaling Technology (Beverly, MA). DNA constructs myr-Akt-pUSE and pUSE empty vector were gifts from Dr. Grant D Stewart from University of Edinburgh. Preparation of Sb culture supernatant (SbS) was done as previously described.9 Briefly, lyophilized Sb (Biocodex Laboratories, Gentilly Cedex, France) was cultured in RPMI 1640 cell culture medium (100 mg/mL) for 24 hours at 37°C. The suspension was then centrifuged at 9000g for 15 minutes and the supernatant collected. The supernatant was then passed through a 0.22-mm filter (Fisher Scientific, Pittsburgh, PA) and then a 10-kilodalton cutoff filter (Millipore, Billerica, MA). For SbS used in ligand-induced signaling experiments, ion exchange chromatography was used during preparation.

**Western Blot Analysis**

HT29, SW480, or HCT-116 cells were incubated with SbS at 1:1 dilution for different time periods at different conditions. Treated cells were then lysed in a lysis buffer (62.5 mmol/L Tris-HCl, 10% glycerol, 2% SDS, 0.01% bromphenol blue, and 1% 2-mercaptoethanol). Equal amounts of cell extract were fractionated by 10% SDS-PAGE, and proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) at 300 mA for 3 hours. Membranes were blocked in 5% nonfat dried milk in TBST (50 mmol/L Tris, pH 7.5, 0.15 mol/L NaCl, 0.05% Tween 20) and then incubated with antibodies directed against phospho- or nonphosphorylated forms of ERK1/2, MEK1/2, HER-2, HER-3, IGF-1R (Cell Signaling), and EGFR (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed with TBST and incubated with horseradish peroxidase-labeled secondary antibodies for 1 hour. The peroxidase signal was detected by Supersignal chemiluminescent substrate (Pierce, Rockford, IL), and the image of the signal was recorded by exposure to x-ray film (Fujifilm, Tokyo, Japan).

**Colony Formation Assay and Cell Proliferation Assays**

HT29 and SW480 cells were seeded at 1000 cells per well in 6-well plates and allowed to attach for 48 hours. SbS was diluted in DMEM at different concentrations and added directly to cell culture wells. Cultures were observed daily for 10–20 days and then were fixed and stained with modified Wright-Giemsa stain (Sigma Chemical Co, St. Louis, MO). Colonies of 30 cells were scored as survivors.16 Cells were maintained at 37°C in 5% CO₂ in complete humidity. HT29 cells were cultured in DMEM supplemented with 10% (vol/vol) FBS and 2 mmol/L L-glutamine ( Gibco). Cell proliferation assays were carried out using both MTS assay kit (Promega, Madison, WI) and BrdU colorimetric kit (Roche Applied Science, Indianapolis, IN) following manufacturers’ instructions.

**Cell Transfection**

Ninety percent to 100% confluent HT29 cells grown in 12-well dishes or 4 chamber polystyrene vessel glass slides were transfected with pUSE empty vector or pUSE-myr-Akt plasmids using Lipofectamine 2000 (Invitrogen, Inc., Carlsbad, CA). Twenty-four hours after transfection, cells were treated with SbS for 30 minutes before being lysed for Western blot analysis or treated over night for TUNEL staining.

**Apoptosis Assays**

Tunel assay was carried out in both HT29 cell culture and intestinal tissues using TUNEL Apoptosis Detection Kit (Upstate Biotechnology Inc., Lake Placid, NY) according to the manufacturer’s instructions. For flow cytometry, adherent HT29 cells were released by treatment with 0.25% trypsin. Each sample was fixed overnight with 70% ethanol at 4°C. Cells were rehydrated with phosphate-buffered saline (PBS) and then stained with 10% propidium iodide in 100 U/mL of ribonuclease in PBS for 60 minutes at room temperature. Cells were filtered through 35-μm filters before analysis. The flow cytometer was configured to track the number of events with the FL2 parameter (FACScan; Becton Dickinson, Lincoln Park, NJ). The DNA content was analyzed using a nonlinear least-squares algorithm. Caspase activation was measured with a fluorimetric homogeneous caspase assay kit (Roche Applied Science).

**Apcm³ Min Mice**

C57BL/6J Min/+ (Apc³Min) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at age 7 weeks. Sb was administrated daily in their drinking water, 3 × 10⁸ colony-forming units (CFU) per milliliter, and 3 times each week by oral gavage at a dose of 6 × 10⁸ CFU for 9 weeks. Mice were killed at 16 weeks of age. Intestinal tumor number was counted in the distal 10 cm of the small intestine using Methylene Blue in PBS (0.05%). Tumor sizes were measured by external caliper and taken as diameter to calculate total areas. Proximal sections of the rest of the small intestine (remaining section after usage of 10-cm distal part) were fixed and used for immunohistochemistry. Grade of dysplasia was measured by a blinded pathologist using a simple grading system based on the criteria for adenomatous change in the human colon: high-grade vs low-grade dysplasia, where low-grade dysplasia showed nuclear elongation with a sessile or villous architecture, and high-grade dysplasia showed a cribriform growth pattern with loss of nuclear
polarity and nuclear rounding. Relevant tissues were fixed overnight in 10% neutral-buffered formalin for immunohistochemistry. Animal studies were approved by the Institutional Animal Care and Use Committee.

**Immunohistochemistry and Imaging Analysis**

Immunohistochemical staining was done on fixed intestinal tissues in Beth Israel Deaconess Medical Center Immunohistochemistry Core Facility using specific antibodies against proliferating cell nuclear antigen (PCNA), phospho-EGFR, and phosphor-Akt and counterstained with hematoxylin. Images were viewed under light microscopy (Eclipse E800; Nikon, Melville, NY) by using a plain Apo 40×/0.95 objective, imported via a SPOT Insight camera (Diagnostic Instruments, Sterling Heights, MI), and stored digitally using SPOT software (Diagnostic Instruments). For semiquantitative analysis of immunohistochemistry imaging, microscopic views of 10 random tumors from each animal group were randomly chosen and examined by a blinded observer. On each microscopic field, cells positively stained by p-EGFR or p-Akt were counted, as well as the total number of cells by hematoxylin staining. In the case of quantifying Tunel-stained colon tumor images, cells positively stained by FITC and total cells by DAPI were counted. The percentage of positively staining cells on each high-power field was calculated for both groups and statistically analyzed.

**Statistical Analyses**

Results were expressed as mean ± SE. Data were analyzed using the SIGMA-STAT professional statistics software program (Jandel Scientific Software, San Rafael, CA). Analyses of variance with protected t test were used for intergroup comparison.

**Results**

**SbS Inactivates EGFR-Mek-Erk Pathway Signaling in Colonic Cancer Cells**

We have reported that Erk1/2 MAP kinase activation is inhibited by SbS in vitro and in vivo.9 We hypothesized that EGFR signaling might be affected upstream to Erk. After varying periods of time (0–180 minutes) of exposure to SbS, HT29 cells were harvested; cell extracts...
were prepared; and Western blotting was performed using either phospho-specific or total EGFR, MEK1/2, ERK1/2 antibodies. We found that SbS markedly reduced EGFR phosphorylation (Tyr1173) with a rapid onset of effect (1 minute). Reversibility of the SbS inhibitory effect was apparent, with some return of EGFR phosphorylation becoming evident at later time points (Figure 1A). Phospho-Mek1/2, a downstream signaling molecule to EGFR but an upstream kinase to Erk1/2, also lost much of its activity after 5 minutes of SbS exposure. As expected, p-Erk1/2 activity was also significantly decreased following dephosphorylation of EGFR and MEK1/2 (Figure 1A). These findings are consistent with the theory that phospho-Erk1/2 is reduced by SbS through effects on the EGFR-Mek-Erk pathway. This provided a mechanism to account for the effects of SbS on Erk1/2 MAP kinase activity as described in our previous studies.9 Because other receptor tyrosine kinases such as IGF-1R and ErbB family members including HER-2 and HER-3 also stimulate MAP kinase signaling, we examined HER-2, HER-3, and IGF-1R activation levels in SbS-treated HCT-116 or HT29 cells. We found that p-HER-2, p-HER-3, and p-IGF-1R are all inactivated by SbS in similar fashion as p-EGFR (Figure 1B). Other signaling kinases, such as phospho-CamKII (Figure 1A) and PKC family members including phosphorylated forms of PKC-č, PKC-č, PKC-β, and PKC-δ were not affected by SbS (data not shown), indicating an effect on receptor tyrosine kinases including EGFR, HER-2, Her-3, and IGF-1R rather than global dephosphorylation and inactivation.

To examine whether SbS inhibits ligand-induced activation of these receptor tyrosine kinases, SW480 or HCT-116 cells were treated with 10 ng/mL recombinant EGF ligand or 100 ng/mL recombinant Neuregulin (NRG-1) ligand, with or without the presence of SbS at various time points. As shown in Figure 1C, SbS completely blocked EGF-induced EGFR activation and attenuated NRG-1-induced HER-2 and HER-3 activation.

**SbS Inhibits Colon Cancer Cell Proliferation and Cell Colony Formation**

The effect of SbS on EGFR signaling led us to examine whether cancer cell proliferation may be altered as a consequence of EGFR deactivation. Using the MTS assay, semi-confluent colon adenocarcinoma HT29 cells were stimulated with 10 ng/mL EGF in the presence or absence of SbS for 24 hours. We found that SbS abolished EGF-stimulated relative cell number increase (P < .001) (Figure 2A). Furthermore, BrdU labeling was used in colorimetric immunoassay for the quantification of cell proliferation. As shown in Figure 2B, SbS significantly reduced EGF-induced cell proliferation (P < .01) quantified by incorporation of BrdU.

We next determined the effect of SbS in cell survival using the colony formation assay. HT29 cells were grown in DMEM containing 10% serum in the absence or presence of different concentrations of SbS for 10–20 days. SbS showed a dose-dependent inhibition of HT29 cell colony formation. At dilutions as low as 1/64, this inhibitory effect was still evident and statistically significant (Figure 2B). Thus, SbS reduces proliferation and colony formation of HT29 cells in vitro. This inhibition was almost identically repeated in SW480 cells treated by the same dilutions of SbS (data not shown).

**SbS Inactivates Phospho-Akt and Induces Cancer Cell Apoptosis**

Because Akt is a key signaling molecule for cell survival and is also a downstream molecule to the EGFR, we next tested whether Akt was also affected by SbS. After varying periods (0–240 minutes) of exposure to SbS, cells were lysed and samples tested by Western blotting for Akt phosphorylation. After 15 minutes of exposure to SbS and, thereafter, Akt phosphorylation was markedly decreased, whereas total Akt remain unchanged (Figure 3A).

In view of these effects on Akt activations, we next determined whether SbS affects cell apoptosis. We
evaluated apoptosis by flow cytometry, caspase assay, and Tunel staining. For flow cytometry, HT29 cells were treated with or without SbS for different time periods (4, 8, 12 hours) and then stained by propidium iodide followed by flow cytometric analysis. We found that SbS treatment increased the sub-G1 cell fraction in a time-dependent manner (control: 12%; SbS treated: 4 hours, 22%; 8 hours, 28%; 12 hours, 40%), suggesting that SbS promoted HT29 cancer cell death (Figure 3B).

Figure 3. SbS inactivates phopho-Akt and induces HT29 cell apoptosis. (A) After varying periods of time (0–240 minutes) of exposure to SbS, HT29 cells were lysed and samples tested by Western blotting using antibodies against total and phopho-Akt. (B) Human colonic cancer cells were treated with SbS for different time periods (4, 8, 12 hours), harvested, and stained by propidium iodide followed by flow cytometric analysis. Cells treated with RPMI for 12 hours were used as a control. Apoptosis was determined by the sub-G1 fraction (in rectangular boxes in Figure). Results are from 1 of 2 independent experiments. (C) HT29 cells treated with SbS for 4 hours at varying dilutions (1/2 to 1/64) were then tested using homogenous caspase assay kit (Roche Applied Science). Cells treated with vehicle serves as negative control. Cells treated with 4 μg/mL camptothecin serve as positive control. Bars represent mean ± SE. P < .01 between SbS-treated groups (at all tested doses) vs negative control. (D) HT29 cells treated with SbS for 12 hours at varying dilutions (1/8, 1/16, 1/32) were tested for apoptosis by Tunel assay using TUNEL apoptosis detection kit. Cells labeled with green fluorescence are those undergoing apoptosis. The average percentage of positively stained cells in those treated with SbS (1/8 dilution) is 32.0% ± 8.5% vs control cells 1.5% ± 0.5% (P < .01). Representative images are shown. (E) Twenty-four hours after HT29 cells were transfected with pUSE empty vector or pUSE-Akt, cells were treated with SbS at 1/2 dilution for 30 minutes then lysed for Western blotting using p-Akt. SbS decreased endogenous p-Akt signal, whereas ectopic myr-Akt remained intact. Cotreatment of cells with EGF (20 ng/mL) reversed the p-Akt signal reduction induced by SbS. (F) HT29 cells transiently transfected with vector or myr-Akt were treated with different dilutions of SbS for 12 hours. Apoptotic cells were then analyzed by Tunel staining. The percentage of Tunel-positive cells was averaged in 3 independent experiments. Both ectopic myr-Akt expression and stimulation by 20 ng/mL EGF ligand reduced SbS-induced apoptosis. Bars represent mean ± SE.
HT29 cells treated with SbS for 4 hours at varying dilutions (1/2 to 1/64) were then tested using a homogenous caspase assay kit (Roche Diagnostics). We found that SbS induced caspase activity in a dose-dependent fashion. Even at the 1/64 dilution, the induction of caspase activity was significantly higher compared with negative control cells (Figure 3C). Furthermore, we demonstrated that SbS induces apoptosis by Tunel staining as shown in Figure 3D. The average percentage of positively stained cells in those treated with SbS (1/8 dilution) is 32.0% ± 8.5% vs control cells 1.5% ± 0.5% (P < .01). These data indicate that SbS inactivates phospho-Akt and induces cancer cell apoptosis.

To confirm the role of SbS-regulated EGFR and Akt in apoptosis, we transiently transfected HT29 cells with constitutively active myr-Akt using the empty pUSE vector as control. Treatment of transfected cells by SbS at 1/2 dilution for 30 minutes decreased endogenous p-Akt signal, whereas ectopic myr-Akt remained intact. Adding EGF (20 ng/mL) to SbS-treated cells reversed the p-Akt signal reduction induced by SbS (Figure 3E). HT29 cells transiently transfected with vector or myr-Akt were treated with different dilutions of SbS for 12 hours, and the apoptotic rate of cells was analyzed by Tunel staining. As shown in Figure 3F, ectopic myr-Akt expression inhibited Sb-mediated apoptosis at all SbS dilutions (P < .01). Stimulation by 20 ng/mL EGF ligand also significantly reduced SbS-induced apoptosis at 1/4 dilution of SbS (P < .01) (Figure 3F).

**Oral Intake of Sb Inhibited Tumor Growth in Apc<sup>Min</sup> Mice**

To test whether Sb inhibits cancer growth in vivo, we administered Sb to C57BL6/min/+ (Apc<sup>Min</sup>) mice daily from age 7 weeks until 16 weeks of age. Mice were then killed, and the 10-cm section of the distal small intestine was harvested, from which we measured the number, size, and grade of dysplasia in intestinal tumors. We found that orally administered Sb led to a significant reduction in the number (mean ± SE: 29.7 ± 4.8 for control [n = 6] vs 16.4 ± 3.0 for Sb treated [n = 8], P = .03), diameter (3.3 ± 0.2 mm vs 2.7 ± 0.2 mm, respectively, P = .03), and total surface area (102 ± 21.7 mm<sup>2</sup> vs 44.5 ± 9.4 mm<sup>2</sup>, respectively, P = .02) of intestinal tumors in the Apc<sup>Min</sup> mice (Figure 4A).

The tumors in the Apc<sup>Min</sup> mice were categorized as showing low- or high-grade dysplasia, using a system based on human colonic carcinoma profiles. Sb-treated mice had a significantly lower score for low-grade dysplasia compared with control mice (P = .01). Scores for high-grade dysplasia were also lower than in the control group, but this difference did not reach statistical significance (P = .12, Figure 4B). Representative images from H&E-stained small intestines demonstrated reduced numbers of polyps (Figure 4C).

Immunohistochemistry using antibodies against PCNA, a cell proliferation marker, showed that Sb-treated Apc<sup>Min</sup> mice had fewer proliferative cells in their tumors (PCNA-positive staining) compared with control mice. Figure 5 shows representative images from both Sb treated Apc<sup>Min</sup> mice and control mice. PCNA-positive cells within the tumor are 66% in control mice, compared with 37% in Sb-treated mice (66% ± 14% vs 37% ± 20%, respectively, P < .01), indicating that cell proliferation is attenuated in tumors of Sb-treated mice (Figure 5A). The average percentages of phospho-EGFR (Tyr1173)-positive cells per high-power field in Sb-treated mice vs control mice are 6.5% ± 2.8% vs 17% ± 5.5%, respectively, P < .05. The average percentages of p-Akt strongly stained cells per high-power field from Sb-treated mice vs control mice are 49.6% ± 5.4% vs 70.4% ± 4.5%, respectively, P = .01. Representative images of p-EGFR and p-Akt in Figure 5B and 5C showed that both activation molecules are less evident in treated mice, indicating that the effects of Sb on phospho-EGFR and phospho-Akt are also evident in vivo, consistent with the results of our in vitro experiments with HT29 cells (Figures 1 and 3A). Tunel staining of intestinal tissues and control Apc<sup>Min</sup> mice demonstrated a significant increase of apoptotic cells from Sb-treated mice. The average percentages of apoptotic cells per high-power field are 15% ± 3.0% in Sb-treated mice vs 4.5% ± 2.3% in control mice, P < .01. Representative images are shown in Figure 5D.

**Discussion**

One of the beneficial effects of Sb is its ability to reduce intestinal inflammatory responses. Erk1/2 MAP kinases, major modulators of host inflammatory responses, are negatively regulated by Sb.5,9 Here, we report that Sb inhibits Erk kinase activity through effects on the EGFR/Mek pathway. The active (phosphorylated) form of EGFR rapidly loses its activity upon exposure to SbS, which leads to the downstream inactivation of p-Mek and p-Erk as well as p-Akt. Dephosphorylation of p-EGFR is not due to a toxic effect of SbS because, as we previously reported, the inhibitory effect of SbS is fully reversible.17 The fact that p-EGFR, p-Mek1/2, and p-Erk1/2 slowly recover their activities, as indicated by our Western blot data, is entirely consistent with this finding.

SbS prevented cancer cell colony formation, reduced EGF-mediated cell proliferation, and increased apoptosis. The in vitro effects are consistent with inhibition of the EGFR and Akt pathways. The inhibitory effect of Sb on p-EGFR and p-Akt appears to occur in vivo also. Immunostaining intensity of both p-EGFR and p-Akt decreased in Sb-treated Apc<sup>Min</sup> mice. Furthermore, Apc<sup>Min</sup> mice treated with Sb demonstrated beneficial effects compared with nontreated mice by showing a 50% decrease in tumor number and a substantial drop in tumor volume. This suggests that Sb may have a beneficial role in preventing or treating intestinal adenomatous polyps and/or adenocarcinoma.
Aberrant activation of EGFR has been shown to be critical for the maintenance of malignancy in a number of solid tumors, including colorectal cancer.\textsuperscript{18,19} Interference with the activation of EGFR and other growth factor receptors represents a promising strategy for novel and selective anticancer therapies.\textsuperscript{20} In the \textit{Apc\textsuperscript{Min}} mouse model, it has been shown that EGFR activity is important in the establishment of intestinal tumors and that \textit{Apc} deficiency is associated with increased EGFR activity.\textsuperscript{21,22} Our in vitro cell signaling studies indicated that SbS inactivates other receptor tyrosine kinases including HER-2, HER-3, and IGF-1R, besides EGFR, which are all involved in cell proliferation and apoptosis and commonly overexpressed in many cancers and play important roles in neoplasia.\textsuperscript{23–25} In our hands, immunohistochemical staining of phospho-IGF-1R yielded no staining pattern in tumors of \textit{Apc\textsuperscript{Min}} mice. However, phospho-EGFR showed clear differences between Sb-treated and control mice. Our data also suggested that both EGF ligand stimulation and constitutively active Akt expression inhibit the proapoptotic effect of SbS, indicating the direct involvement of EGFR-Akt pathway in Sb-mediated apoptosis. The roles of IGF-1R and HER-2 and -3 in Sb-mediated cell proliferation and apoptosis, as well as tumor reduction in \textit{Apc\textsuperscript{Min}} mice, need to be further addressed in our future studies. However, the inhibitory effect of Sb on EGFR activity, with downstream effects on Akt, at least in part explains its beneficial effect in reducing tumor growth.

As yet, the detailed mechanism of how SbS inactivates both basal and ligand-induced activation of EGFR and HER-2 and -3 is unknown. Compartmentalization of receptor tyrosine kinases including EGFR into subdomains of the cell membrane is an important control
Figure 5. Immunohistochemical staining showing the in vivo effect of Sb on EGFR and Akt phosphorylation and on cell proliferation in the intestinal tumors of Apc\textsuperscript{Min} mice. (A) The murine small intestines were stained using PCNA antibodies and counterstained with hematoxylin. Dark/black colored nuclear staining indicates PCNA-positive cells. Cells with blue nuclei are PCNA negative. Semiquantitative analysis using 5 representative images from each group showed that tumors from Apc\textsuperscript{Min} mice treated with Sb possess fewer proliferative cells compared with those from control mice (37% ± 20% vs 66% ± 14%, respectively, P < .01). (B and C) Antibodies against phospho-EGFR (Y1173) and phospho-Akt were used to stain the intestinal tumors counterstained with hematoxylin. From microscopic views of 10 tumors from each group, the percentage of phospho-EGFR (Tyr1173)-positive cells per high-power field in Sb-treated mice vs control mice are 6.5% ± 2.8% vs 17% ± 5.5%, respectively (mean ± SE, P < .05). The average percentage of p-Akt strongly stained cells per high-power field from Sb-treated mice vs control mice is 49.6% ± 5.4% vs 70.4% ± 4.5%, respectively, P = .01. Representative images are shown to compare the different staining intensity for p-EGFR and p-Akt between treated and control Apc\textsuperscript{Min} mice (brown shows phospho-EGFR or phospho-Akt). (D) Tunel staining of intestinal tissues from Sb-treated and control Apc min mice. FITC (green)-labeled cells are apoptotic tumor cells with DAPI (blue) staining as background. From 10 microscopic views each from the treated and control groups, the average percentages of apoptotic cells per high-power field are 15% ± 3.0% in Sb-treated mice vs 4.5% ± 2.3% in control mice (mean ± SE, P < .01). Representative Tunel staining images are shown with red arrow pointing to apoptotic cells within the tumors.
mechanism for signaling. One hypothesis is that SBs may alter the membrane localization of receptor tyrosine kinases and their association with lipid rafts. This may explain the effects of SB on a variety of receptor tyrosine kinases. Nonetheless, our findings indicate that the yeast SB can exert anti-cancer effects through inactivation of growth receptors. Receptor tyrosine kinases are key molecular targets for cancer therapy, and, to our knowledge, this is the first report of major receptor tyrosine kinase inactivation by a probiotic microorganism. Several animal studies showed a reduction in chemically induced colorectal tumor incidence accompanying probiotic bacteria administration; however, the mechanisms were not well understood. Our findings that SB inhibits EGFR signaling and reduces tumor growth in Apcmin mice provide a novel mechanism for probiotic actions against cancer. SB appears to exert its beneficial effects by multiple potential mechanisms of action, including neutralization of bacterial virulence factors, interference with bacterial adhesion, strengthening of enterocyte tight junctions, enhancement of the mucosal immune response, altering immune cell redistribution, and modulating inflammatory signaling pathways of the host. Given the complicated picture of probiotic actions, we cannot exclude other potential mechanisms that may explain the anti-cancer phenomenon of SB. Besides receptor tyrosine kinase inactivation, the anti-inflammatory properties of SB or possible gut flora changes because of SB administration in Apcmin mice may also impact intestinal tumor development. Further studies are needed to explore these potential mechanisms. Nevertheless, with decades of usage profile in treating gastrointestinal disorders, the clinical effects of SB on prevention and treatment of colon polyps and colon cancer clearly warrant further investigation.

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The authors disclose no conflicts.

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