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Citation: Lakritz, Jessica R., Theofilos Poutahidis, Tatiana Levkovich, Bernard J. Varian, Yassin M. Ibrahim, Antonis Chatzigiagkos, Sheyla Mirabal, Eric J. Alm, and Susan E. Erdman. "Beneficial Bacteria Stimulate Host Immune Cells to Counteract Dietary and Genetic Predisposition to Mammary Cancer in Mice." *Int. J. Cancer* 135, no. 3 (January 10, 2014): 529–540.

As Published: <http://dx.doi.org/10.1002/ijc.28702>

Publisher: Wiley Blackwell

Persistent URL: <http://hdl.handle.net/1721.1/96512>

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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Beneficial bacteria stimulate host immune cells to counteract dietary and genetic predisposition to mammary cancer in mice

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Recent studies suggest health benefits including protection from cancer after eating fermented foods such as probiotic yogurt, though the mechanisms are not well understood. Here we tested mechanistic hypotheses using two different animal models: the first model studied development of mammary cancer when eating a Westernized diet, and the second studied animals with a genetic predilection to breast cancer. For the first model, outbred Swiss mice were fed a Westernized chow putting them at increased risk for development of mammary tumors. In this Westernized diet model, mammary carcinogenesis was inhibited by routine exposure to *Lactobacillus reuteri* ATCC-PTA-6475 in drinking water. The second model was FVB strain erbB2 (HER2) mutant mice, genetically susceptible to mammary tumors mimicking breast cancers in humans, being fed a regular (non-Westernized) chow diet. We found that oral supplement with these purified lactic acid bacteria alone was sufficient to inhibit features of mammary neoplasia in both models. The protective mechanism was determined to be microbially-triggered CD4+CD25+ lymphocytes. When isolated and transplanted into other subjects, these *L. reuteri*-stimulated lymphocytes were sufficient to convey transplantable anti-cancer protection in the cell recipient animals. These data demonstrate that host immune responses to environmental microbes significantly impact and inhibit cancer progression in distal tissues such as mammary glands, even in genetically susceptible mice. This leads us to conclude that consuming fermentative microbes such as *L. reuteri* may offer a tractable public health approach to help counteract the accumulated dietary and genetic carcinogenic events integral in the Westernized diet and lifestyle.

Breast cancer is the most prevalent type of cancer in women worldwide and a leading cause of neoplasia-associated mortality.¹ Although genetic predisposition plays a role, accumulating epidemiological and experimental data also link an unhealthy diet and obesity with postmenopausal mammary

Key words: mammary cancer, probiotics, bacteria, inflammation, *L. reuteri*

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Grant sponsor: National Institutes of Health; **Grant numbers:** P30-ES002109, RO1CA108854; U01 CA164337

DOI: 10.1002/ijc.28702

History: Received 5 July 2013; Accepted 23 Dec 2013; Online 31 Dec 2013

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cancer occurrence and growth.^{1–6} The mechanisms explaining this connection are largely unknown. Several lines of evidence, however, suggest that inter-related metabolic and inflammatory pathways may be involved. Specifically, altered hormonal pathways involving estrogens, insulin and insulin growth factor, imbalances in the expression of adipokines such as leptin and adiponectin and obesity-associated proinflammatory signaling have all been shown to promote mammary tumorigenesis.^{1,4–10} Interestingly, data from several elegant experiments using high-fat diet-fed mice indicate that obesity-associated inflammation could promote mammary cancer independently from ovarian hormones,^{8,10} insulin⁶ and adipokine effects.⁹ Taken together these data suggest that immune system-related events may hold the key trigger role in the complex interplay between growth factors, hormones and adipokine signaling, which connects diet and obesity with mammary carcinogenesis.¹¹ Despite the progress made in identifying the mechanisms underlying the obesity-mammary cancer associations, the question “how do we block the adverse effects of obesity on postmenopausal breast cancer?” has been recently characterized as urgent.¹¹

In an earlier series of experiments we have shown that inflammatory signals from bacteria colonizing the gut can trigger mammary carcinogenesis in mice.^{12–14} Specifically, anti-inflammatory therapies including anti-tumor necrosis

What's new?

Can eating fermented foods like yogurt ward off cancer? Recent studies have suggested it's possible. To find out how, these authors isolated the probiotic bacteria involved in fermentation and fed them to mice that were susceptible to mammary tumors. These mice either had genetic changes predisposing them to cancer or were fed a diet known to increase their likelihood of developing the tumors. Eating the lactic acid bacteria stimulated an immune response that delayed the onset of tumors in both models, suggesting a potential avenue to prevent the cancer in humans.

factor (TNF)-alpha treatments, and adoptive cell transfer using gut-bacteria-activated regulatory T (Treg) cells are able to block tumor-promoting effects in both ApcMin^{+/+} and MMTV-HER2/neu mouse model.^{12–14} Murine models exhibiting mutations in the *erbB2* gene are widely used to mimic neoplastic processes seen in ~30% of patients with breast cancer.¹⁵ Interestingly, recent evidence from women with estrogen-receptor-negative breast cancer shows that tumor-infiltrating Treg are associated with a more favorable clinical outcome.¹⁶

In a more recent series of experiments we discovered that feeding mice with the edible probiotic bacterium *Lactobacillus reuteri* (*L. reuteri*) exerts unexpected effects in the integument, namely that *L. reuteri*-fed mice have radiant fur with increased anagenic hair follicles.¹⁷ In these studies *L. reuteri* was chosen due to broad relevancy by colonizing many mammalian species, the precedent for *L. reuteri* to treat or prevent human diseases such as diarrhea in children, the well-established precedent in scientific literature using *L. reuteri* as a model probiotic organism, that *L. reuteri* ATCC-PTA-6475 was originally isolated from human milk, previously demonstrated efficacy in inflammatory diseases in mouse models, in particular this “anti-inflammatory” isolate suited our immune system study goals, and *L. reuteri* is very easy to cultivate and dose to animals. In a separate line of experiments we find that *L. reuteri* rescues mice from diet-induced obesity and fat pathology via CD4⁺Foxp3⁺CD25⁺ regulatory T (Treg) cell-associated immunomodulatory effects.¹⁸ Our recent findings regarding the effects of *L. reuteri* upon Treg cells are in line with previous studies showing that *L. reuteri* stimulates the development of host protective regulatory-T cells *in vitro* as well as *in vivo*.^{19–21} These data taken together with previous evidence that dietary lactic acid bacteria could lower the risk of breast cancer in women,^{22,23} and also suppress mammary cancer development in mice through immune-system regulatory events,^{24–26} led us to test the effects of *L. reuteri* consumption in mouse models of mammary cancer.

In the present study, using first a high-fat diet-associated outbred mouse, and then a classical, diet-unrelated genetically engineered MMTV-HER2/neu mouse model of mammary cancer, we found that eating purified lactic acid bacteria alone was sufficient to delay onset of preneoplastic or neoplastic features in both models. The protective mechanism involved microbially triggered antigen activated CD4⁺CD25⁺ cells that were sufficient to bestow transplantable anti-cancer protection to recipient host MMTV-HER2/neu mice. These

data demonstrate that host immune responses to environmental microbes significantly impact cancer progression in distal nonintestinal tissues. In these studies, modulation of the tumor micro-environment by regulating systemic immune cell responses at the whole organism level was found to counteract dietary or genetic predisposition to cancer.

Material and Methods**Animals**

Outbred CD-1 mice (Charles River; Wilmington, MA), and inbred MMTV-*neu* (*HER2*) FVB strain mice (Jackson Labs, Bar Harbor, ME), were housed and handled in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facilities with diets, experimental methods, and housing as specifically approved by the Institutional Animal Care and Use Committee. The MIT CAC (IACUC) specifically approved the studies as well as the housing and handling of these animals. For studies using outbred mice, the experimental design was to expose mice to diets starting at age = 8 weeks, and then continue the treatment for 12 weeks until euthanasia using carbon dioxide overdose at 5 months of age, unless otherwise specified. Each experiment included 5–15 animals per group with one replicate experiment. Mammary tissues were collected upon necropsy and then examined histologically.

For experiments using HER2 mice, age-matched female HER2/*neu* mutant mice were either untreated or had their water continuously supplemented with *L. reuteri* beginning at 8 weeks of age. Mice were euthanized when animals reached 1 year of age, or earlier if the total cumulative tumor burden on the animal reached 2 cm in diameter. In studies using adoptive cell transfer, otherwise untreated 6-month-old female HER2 mice with pre-existing tumor burden were randomly subdivided into treatment groups that received cell transplant injections as described in detail below.

Special diets for animals

Outbred Swiss mice were placed on experimental diets starting at 8 weeks of age: control AIN-76A (Harlan–Teklad Madison WI), and a Westernized diet with high fat and low fiber with substandard levels of Vitamin D (TD.96096; Harlan–Teklad), consumed until euthanasia at 20 weeks of age.

In a separate set of studies, FVB strain HER2 transgenic mice and FVB strain wt cell donor animals received a standard animal facility mouse chow (Pro Lab RMH 3000; Purina, Richmond IN).

Subsets of these Swiss or FVB mice received in their drinking water an anti-inflammatory strain of *Lactobacillus reuteri* ATCC-PTA-6475 cultivated as described elsewhere,^{17,27} with live organisms supplied at a starting dosage of 3.5×10^5 organisms/mouse/day in drinking water. Live bacterial counts in water bottles were calculated to be 1.4×10^6 colony forming units (CFU) per mouse after 24 hr, 4.1×10^5 CFU at 48 hr, and 1.1×10^5 CFU at 72 hr, when quantified as described in detail below. Control mice received regular drinking water. Fresh drinking water for both groups of animals was replaced twice weekly throughout the experiments. Levels of *L. reuteri* were undetectably low in untreated animals using PCR as described below.

Confirmation of *L. reuteri* organisms in drinking water

Lactobacillus reuteri was cultivated as described in previous studies.^{17,27} To ascertain counts of live organisms in drinking water per day, log phase cultures were diluted to 0.05 OD and 100 μ L plated for overnight growth on blood agar plates (BAP). Overnight cultures were collected into sterile phosphate buffered saline (PBS) and the optical density (OD) was measured. The OD of the preparation was adjusted to 1.0 and measured once again for confirmation. Then 1 mL of suspension was added to five separate bottles containing 480 mL of sterile water each and allowed to incubate overnight. The following morning the contents were mixed by gentle inversion and 1 mL was diluted 100-fold in sterile water; 50 μ L of this dilution were plated at a frequency of 3 BAP per bottle. Again bottles were allowed to stand overnight and another 1 mL sample taken from them each, then diluted 100-fold and plated. This process was repeated over 4 days. Colonies were counted 48 hr after plating due to small colony size. Counts were averaged across and between bottles per day and number of total CFU in drinking water was extrapolated based on dilution fold and total water volume.

Confirmation of *L. reuteri* organisms in experimental animals

Feces were collected from animals prior to *L. reuteri* treatment as well as at time of necropsy to gauge its presence post-treatment in comparison with baseline levels of the bacteria. DNA was extracted using the QIAamp DNA Stool Mini Kit from Qiagen. The extraction was performed as per the manufacturer's specifications with the exemption of an added fecal dissociation step during the resuspension where sterile beads were added to the samples and subsequently placed in a Bullet Blender (New England Biogroup) for more efficient sample homogenization. *L. reuteri* specific quantitative PCRs were performed utilizing primers reported by Dommels *et al.*²⁸ on an Applied Biosystems 7500 real-time PCR machine at a final concentration of 0.4 μ M each. Assays were conducted in 25 μ L volumes containing 12.5 μ L SYBR Select Master Mix (Applied Biosystems). DNA concentrations were measured utilizing a Thermo Science Nanodrop 2000C and 100 ng of DNA were loaded per reaction. Amplification was performed verbatim as previously specified²⁸ and abso-

lute quantifications were performed by comparison to a dilution series (data not shown).

Depletion of CD25⁺ cells

Mice were treated with anti-CD25 antibody (clone PC-61; Bio-Express, West Lebanon, NH) at 150 μ g per mouse intraperitoneally 3 \times weekly for 12 weeks starting a 8-weeks-of-age. Treated mice were compared to mice receiving sham isotype antibody alone. Depletion of CD25⁺ cells was confirmed by undetectably low fractions of CD25⁺ cells in spleens of mice treated with anti-CD25 antibody compared to sham-treated controls using flow cytometry. Depletion was confirmed by absence of Foxp3⁺ cells in spleen.

Adoptive transfer of T cells into recipient mice

CD4⁺ lymphocytes isolated from *wild type* FVB strain female mice using magnetic beads (Dyna/Invitrogen; Carlsbad CA) are sorted by hi-speed flow cytometry (MoFlow2) to first obtain purified populations of CD4⁺ lymphocytes and determined to be \sim 96% pure as previously described elsewhere.¹⁴ Syngeneic FVB strain MMTV-neu transgenic recipient mice were then injected intraperitoneally with 3×10^5 CD4⁺ CD45RB¹⁰CD25⁺ cells determined to be \sim 96% pure as previously described elsewhere.¹⁴

Histopathology and immunohistochemistry

For histologic evaluation, formalin-fixed tissues were embedded in paraffin, cut at 5 μ m, and stained with hematoxylin and eosin. Lesions were analyzed and quantified by a pathologist blinded to sample identity. Hyperplastic and preneoplastic lesions in the mammary gland of aged Swiss mice were scored on a 0–6 scale according to the following scheme: 0: normal; 1: Focal, multifocal or diffuse hyperplasia, low grade without atypia; 2: Focal, multifocal or diffuse hyperplasia, intermediate grade without atypia; 3: Focal, multifocal or diffuse hyperplasia, high grade without atypia; 4: Focal, multifocal or diffuse hyperplasia with mild atypia; 5: Focal, multifocal or diffuse hyperplasia with marked atypia; 6: Carcinoma *in situ*.

Immunohistochemistry (IHC) and morphometric assessment were as previously described.¹⁴ IHC-positive immune cells or pixels and toluidine-stained mast cells were counted in 20 \times or 40 \times images and results were recorded as number of cells or pixels per image. Primary antibodies for IHC included rabbit polyclonal antibodies for β -catenin (ThermoFisher Scientific/Lab Vision, Fremont, CA), cleaved caspase-3, NF κ -B-p65 and c-Jun (Cell Signaling, Beverly, MA), Ki-67 (Cell Marque, Rocklin, CA), and a rat monoclonal antibody for Foxp3 (eBioscience, San Diego, CA) detection. Primary antibody binding was detected with goat anti-rabbit polymer HRP (ZytoChem Plus, Berlin, Germany) or biotinylated goat anti-rat IgG (Serotec, Oxford, UK). Heat-induced antigen retrieval was performed with citrate buffer, pH 6, for β -catenin, cleaved caspase-3, NF κ -B-p65 and c-Jun or with EDTA buffer, pH 8, for ki-67 and Foxp-

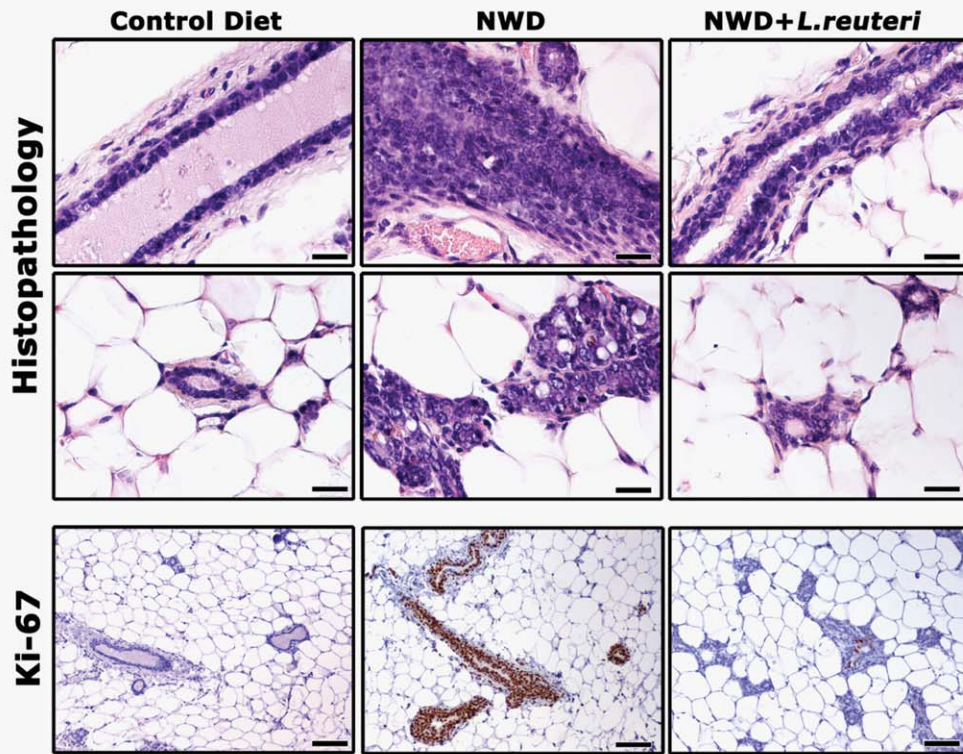


Figure 1. Effect of diet and *L. reuteri* supplementation on mammary gland pathology of Swiss mice. Ducts (upper row) and the terminal duct lobular units (middle row) of westernized diet-fed mice have precancerous epithelial lesions. The mammary intraepithelial neoplasia cells fill the lumen of the duct and show increased mitoses, and marked pleomorphism. The lesion in mammary alveoli is an atypical lobule showing histological features of the “hyperplastic alveolar nodules” such as atypical cells, an intermediate grade of anisokaryosis and light-brown stained lipid pigments. Compare with normal histology of mammary glands from mice consuming control diet or westernized-diet+*L. reuteri*. The westernized diet-induced hyperproliferative state of mammary gland epithelium can be better appreciated with ki-67 specific immunohistochemistry. Ki-67+ proliferating cell nuclei are numerous in contrast to control-diet or westernized-diet+*L. reuteri*-fed mice. Histopathology: Hematoxylin and eosin. Ki-67: DAB chromogen, Hematoxylin counterstain. Bars: Histopathology = 50 μm ; Ki-67 = 100 μm .

3. The ImageJ image processing and analysis program (NIH, Bethesda, MD) was used for all quantitative histomorphometry assessments.

Statistical analyses

The Mann-Whitney U test was used for body weight, and histomorphometry. Mammary tumor volume and multiplicity was evaluated using unpaired student *T* test. A *p* value < 0.05 was statistically significant.

Results

Outbred Swiss mice eating Westernized ‘fast food’ style diet have increased risk of mammary preneoplastic lesions

Obesity-inducing diets promote mammary cancer in both humans and mice.^{1–6} To recapitulate these observations we fed genetically outbred Swiss female mice an *ad libitum* diet of New Westernized diet (NWD) chow mimicking typical human “fast food”-style diets that are high in fat and sugar, and low in fiber and vitamins B and D. We examined the mammary tissues of the 5-month-old mice when fed continuously on Western (*N* = 10 animals) and control (*N* = 10 animals) diets

starting at age = 8 weeks, in order to determine the frequency and spectrum of diet-induced mammary pathology. We found that the mammary ducts and terminal duct lobular units of Westernized chow-fed mice had increased focal, multifocal, or diffuse hyperplastic lesions of varying severity. Hyperplastic glands often showed lateral budding, epithelial cell atypia, increased mitotic figures and intra-epithelial neoplasia (carcinoma *in situ*). Foci of hyperplastic alveoli resembling hyperplastic alveolar nodules (HAN) were also evident (Fig. 1). The evidence of proliferating cells with ki-67-specific immunohistochemistry confirmed the hyperproliferation of mammary glands in mice eating Western chow (Fig. 1). Using a semi-quantitative scoring system (as shown in Fig. 2a), we found that the mammary pathology score of Westernized chow-fed mice differed significantly (*p* < 0.001) from that of mice consuming control diet (Figs. 1 and 2b).

Western chow-induced mammary preneoplasia leads to overt mammary neoplasia

To examine the malignant potential of diet-associated mammary preneoplasia and early neoplasia found at Swiss mice at

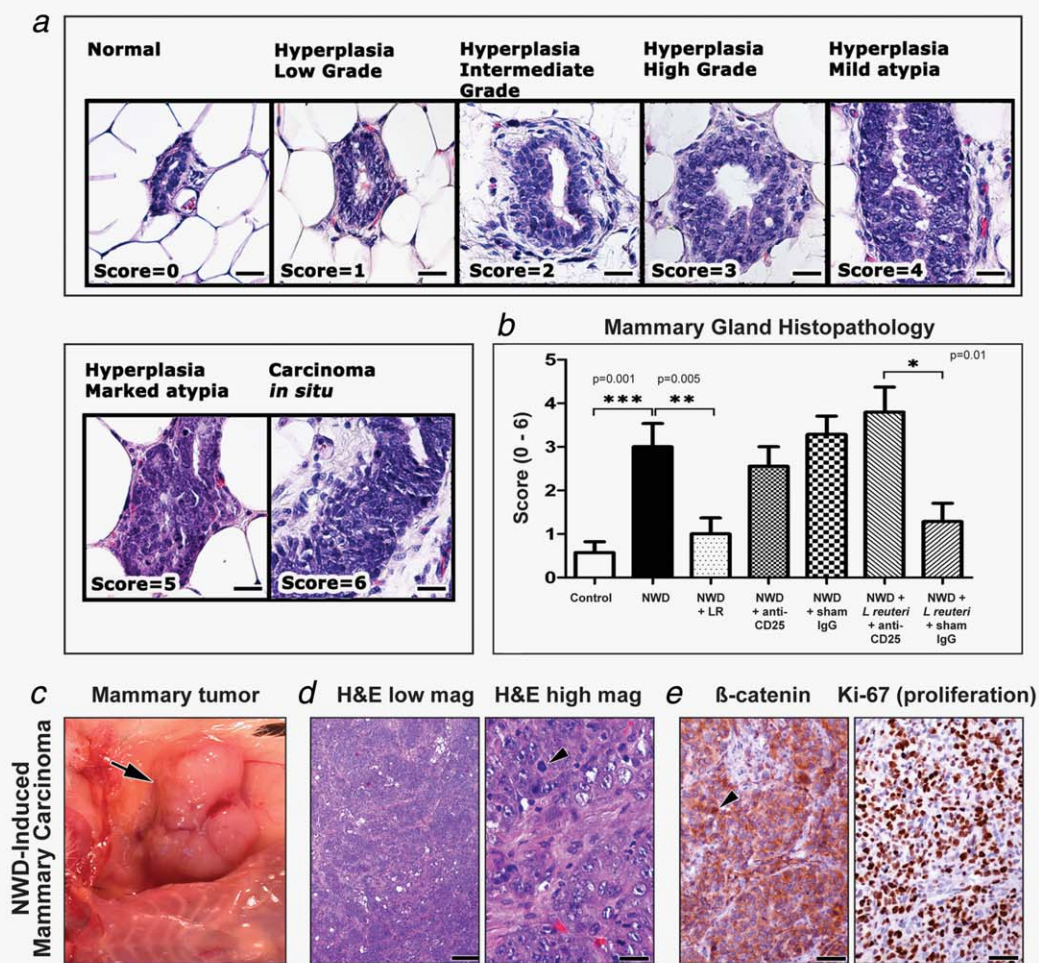


Figure 2. Westernized diet-induced mammary carcinogenesis in Swiss mice. (a) Progression of diet-associated hyperplastic and early neoplastic lesions in the mammary glands of 5-months-old Swiss mice. Figures demonstrate the grading system and depict the spectrum of epithelial histopathology. Note the advancement of hyperplasia and the progressively increased cellular atypia, typified by increased nuclear sizes and pleomorphism. (b) Statistical analysis of semi-quantified mammary gland histopathology. The y-axis depicts the mean \pm SEM of histopathological scores given in each experimental group. The westernized-diet significantly promotes mammary gland pathology, *L. reuteri* restores it and depletion of CD25⁺ cells neutralizes the *L. reuteri* beneficial effect. (c) Gross pathology of mammary tumors (arrow) found in westernized diet-fed Swiss mice with the progression of age. (d) Typical histopathology of diet-associated overt mammary tumors of aged Swiss mice. Variably sized neoplastic glands grow in cords and nests and have a medium amount of intervening stroma. Malignant epithelial cells are highly atypical with marked nuclear pleomorphism, increased mitotic figures, including atypical ones (arrow), and apoptosis. (e) The mammary tumors show immunohistochemical evidence of abnormal β -catenin distribution (left panel). There is loss of the normal lateral membrane staining pattern, diffuse cytoplasmic and occasional nuclear stabilization (arrow-head). Numerous Ki-67⁺ (proliferating) neoplastic cells are shown in the right panel. (a and d) Hematoxylin and eosin. (e) DAB chromogen, hematoxylin counterstain. Bars: a and d-right panel = 25 μ m; d-left panel = 100 μ m; e = 50 μ m.

5 months of age, we assessed the frequency of mammary tumors arising in these mice with the progression of age. We discovered 2-cm diameter mammary tumors (Fig. 2c) arising in two (2/6; 33%) of 1-year-old Swiss mice on Westernized chow, but none in their age-matched female Swiss mice consuming a control diet (0/20). Overt mammary tumors in these mice eating Western chow exhibited typical histopathology of high-grade glandular adenocarcinoma (Fig. 2d). In addition to traditional criteria of high mitotic index and cellular atypia, malignant phenotype of these tumors was further confirmed by immunohistochemistry profiles consistent with carcinoma (Fig. 2e). The neoplastic cells had an abnormal

β -catenin staining pattern with diffuse cytoplasmic and occasional nuclear stabilization, indicative of dysregulation in the *wnt* gene signaling pathway. Proliferating ki-67⁺ neoplastic cells were numerous throughout mammary tissue (Fig. 2e).

Western diet-associated mammary carcinogenesis occurs in a mast cell enriched environment

It is widely known that intestinal microbes modulate host health through activities of CD4⁺ immune cells, at least in part through interleukin (IL)-6-dependent reciprocal functions of anti-inflammatory Foxp3⁺ regulatory (Treg) cells and proinflammatory T helper (Th) 17 cells. Based upon our

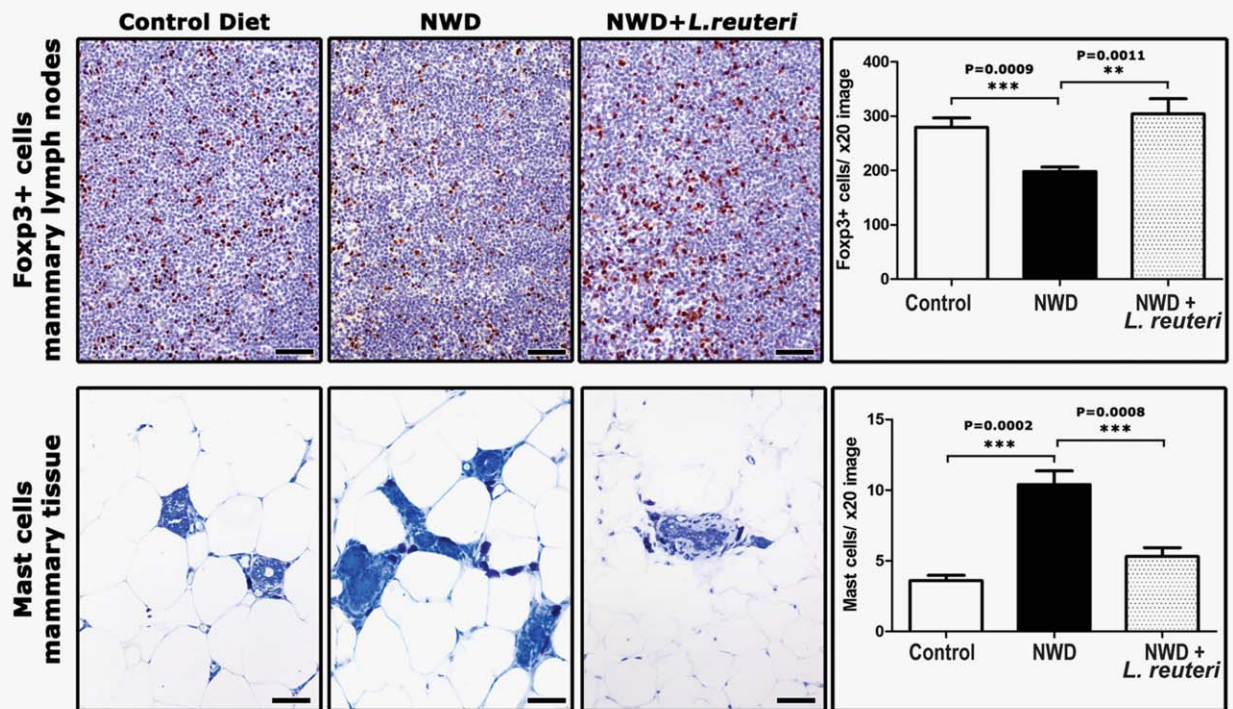


Figure 3. The *L. reuteri* anti-neoplastic effect correlates with restoration of control-level Treg and mast cell populations. Western-type diet induced lesions in the mammary gland correlate with a morphometrically assessed significant decrease of Foxp3+ in the mammary lymph nodes and significant increase of peri-glandular Toluidine Blue-stained mast cells. Dietary supplementation with *L. reuteri* restores the immune cell populations along with mammary gland histology at the base-line control diet-matched levels. Foxp3-specific IHC: DAB chromogen, Hematoxylin counterstain. Mast cells: Toluidine Blue stain. Bars = 50 μ m.

previous observations involving inflammatory cells and cytokines in mouse models of cancer,^{12–14} we examined critical cellular components of the immune system of the 5-month-old Westernized “fast food”-style Westernized diet-fed Swiss female mice. At this time-point of neoplastic initiation, we found that the mammary lymph nodes of NWD-fed mice had significantly fewer Foxp3+ cells compared to the mammary lymph nodes of mice consuming the control diet (Fig. 3). This result matches our previous findings regarding the effect of NWD on the mesenteric lymph node Treg cell population. In the NWD-fed female Swiss mice, a topographical association of Foxp3+ cells with abnormal mammary glands was not observed. Abnormal glands, however, were surrounded by significantly ($p < 0.0002$) increased numbers of mast cells, as previously shown in mammary carcinoma of humans and rodent models.²⁹ Consequently, the number of mast cells in the abnormal mammary epithelium of Westernized chow-eating mice was significantly higher from that of control diet mice, accompanied by sparse Foxp3+ Treg cells (Fig. 3).

Western diet-induced neoplastic features were reduced after consuming purified *L. reuteri* organisms

Previous studies have shown that routine dietary intake of lactic acid bacteria may lower the risk of breast cancer in women,^{22,23} and also suppress mammary cancer development

in mice.^{24–26} This led us to test the effects of *L. reuteri* consumption in this mouse model of mammary malignancy ($N = 12–15$ animals per group). We found that hyperplastic and pre-neoplastic features were observed in significantly ($p < 0.005$) lower frequency in animals receiving purified *L. reuteri* ATCC-PTA-6475^{17,27} when adding 3.5×10^5 organisms/mouse/day to their regular drinking water (Figs. 1 and 2b). *L. reuteri*-induced restoration of glandular epithelium coincided with a significant increase of mammary lymph node Foxp3+ cells and a significant reduction of mammary tissue mast cells to the base-line control-diet levels (Fig. 3).

We next examined whether consumption of *L. reuteri* may inhibit Western diet-induced obesity. Accumulating data specifically link Westernized diet-induced obesity with mammary cancer occurrence and growth.^{1–6} Existing data suggest that immune system-related events may hold the key trigger role in the complex interplay between growth factors, hormones and adipokine signaling, which connects high fat diet and obesity with mammary carcinogenesis.¹¹ We found that 5-month-old mice eating NWD and also consuming *L. reuteri* in drinking water were significantly leaner than those mice fed NWD alone (Supporting Information Fig. S1). We had previously found that while Swiss mice eating *L. reuteri* were uniformly slender, their age-matched counterparts eating *L. reuteri* and simultaneously treated with anti-CD25 antibody instead rapidly developed morbid obesity and

profound abdominal fat pathology during the ensuing 3 months.¹⁸ Swiss mice eating *L. reuteri* also exhibited significantly increased anti-inflammatory Foxp3⁺ Treg cells and lower levels of IL-17 protein within lymph tissues draining the intestinal tract, along with higher levels of IL-10 protein and lower levels of IL-17 protein in serum, when compared with matched control mice.¹⁸ This suggested that host ability to recruit properly functioning anti-inflammatory Treg cells and resulting immune homeostasis is essential for protection from obesity. Following this reasoning, we postulated that intestinal microbe-activated CD4⁺CD25⁺ Treg cells may serve to connect obesity with risk for development of cancer in tissues such as mammary gland.

Anti-neoplastic effect of *L. reuteri* requires antigen-educated CD25⁺ host immune cells

Based upon our earlier work linking intestinal bacteria with mammary glands *via* recruitment of CD4⁺CD25⁺ immune cells,^{13,30} plus other studies showing dietary lactic acid bacteria suppress mammary cancer through immune-system regulatory events,^{24–26} we hypothesized that lactic acid organisms such as *L. reuteri* may protect from cancer by favorably biasing the immune system through induction of anti-inflammatory CD4⁺CD25⁺ Treg cells. We tested this using depletion of CD4⁺CD25⁺ cells *via* systemic anti-CD25 antibody administration in *L. reuteri*-treated mice ($N=6$ animals). We found that anti-CD25 systemic treatment negated the beneficial effect of *L. reuteri*, and resulted instead in increased mammary hyperplastic and preneoplastic lesions (Figs. 2b and 4) with an abnormal mammary gland-associated accumulation ($p<0.002$) of mast cells (Fig. 4). The latter mast cell accumulation effect was also observed in untreated Westernized diet-fed mice depleted of CD25⁺ cells and without *L. reuteri* (Supporting Information Fig. S2). This result further supports the conclusion that the systemic CD25⁺ cell population inversely correlates with the mast cell accumulations in the mammary gland. In sum, the female mice fed Westernized chow were susceptible to obesity and mast cell-associated mammary carcinoma that were inhibited by feeding *L. reuteri*. The protective effect of consuming *L. reuteri* occurred in a CD25 cell-dependent manner.

Mammary tumor development is independent of obesity in MMTV-neu HER2 transgenic mice

To test whether an obesity component is specifically required for cancer-protective benefits of *L. reuteri*, we next tested *L. reuteri* efficacy in mice that do not typically develop age-associated obesity: an FVB strain erbB2 mutant mouse genetically predisposed to mammary tumors mimicking 30% of breast cancers diagnosed in women. For these studies, age-matched HER2/neu mutant mice, that develop tumors without obesity when eating regular chow, were either untreated or had their drinking water supplemented with *L. reuteri*. The study duration allowed mice to reach 1 year of age, unless these animals were humanely euthanized prematurely

due to a total cumulative mammary tumor burden exceeding 2 cm in diameter. The HER2 transgenic mouse model, unlike the outbred Swiss mice which were obese, did not require age-associated obesity in order to spontaneously develop numerous and large-sized mammary tumors (Fig. 5 and Supporting Information Fig. S3).

Tumor-free survival was increased in HER2 transgenic mice after eating purified *L. reuteri*

Knowing that dietary intake of certain lactic acid organisms was previously shown to lower the risk of breast cancer in women,^{22,23} and mammary cancer in mice,^{24–26} led us to test the effects of *L. reuteri* consumption in mouse models of mammary malignancy. Age-matched HER2/neu mutant mice were either untreated or had their water supplemented with *L. reuteri* ATCC-PTA-6475^{22,33} with live organisms supplied at a starting dosage of 3.5×10^5 organisms/mouse/day in drinking water. Mice were euthanized when the total cumulative tumor burden on the animal reached 2 cm in diameter or when animals reached 1 year of age. We found that treating with *L. reuteri* in water delayed or completely prevented tumor onset when compared with untreated mice (Fig. 5a). This showed that consumption of lactic acid bacteria alone was sufficient to inhibit mammary tumorigenesis and improve tumor-free longevity, even in mice genetically predisposed to cancer, with consumption of lactic acid microbes counteracting genetic risks.

Apoptosis was upregulated in MMTV-neu mammary tissues after eating *L. reuteri*

In addition, we found that key indicators of malignancy were significantly altered in transgenic animals receiving regular chow in combination with purified *L. reuteri* in their drinking water (Fig. 6 and Supporting Information Fig. S4). Mammary tumors of untreated MMTV-neu HER2 mice ($N=10$ animals) eating regular diets displayed the typical histopathological features for this mouse model. Tumors were primarily solid (Supporting Information Fig. S4) but often had areas displaying a papillary growth pattern (not shown). In contrast to spontaneous tumors of aged Swiss mice on westernized diet, HER2/neu tumors showed no abnormal β -catenin staining pattern (Supporting Information Fig. S5). Dietary *L. reuteri* in HER2 mice ($N=8$ animals) did not alter the histopathological pattern of tumor growth or the proliferating capacity of tumor cells. However, tumors from probiotic-fed mice had increased areas of intra-tumoral necrosis and increased features of histopathologically discernible apoptosis. To quantify apoptosis more accurately we utilized caspase-3 specific immunostaining. Morphometric counts of apoptosis showed that tumors from *L. reuteri*-treated mice had significantly ($p<0.0021$) more apoptosis compared to tumors from their nontreated counterparts. To further elaborate on this result, we examined the tumors for the expression of two pleiotropic factors with known anti-apoptotic effects on tumors, including those originating from mammary

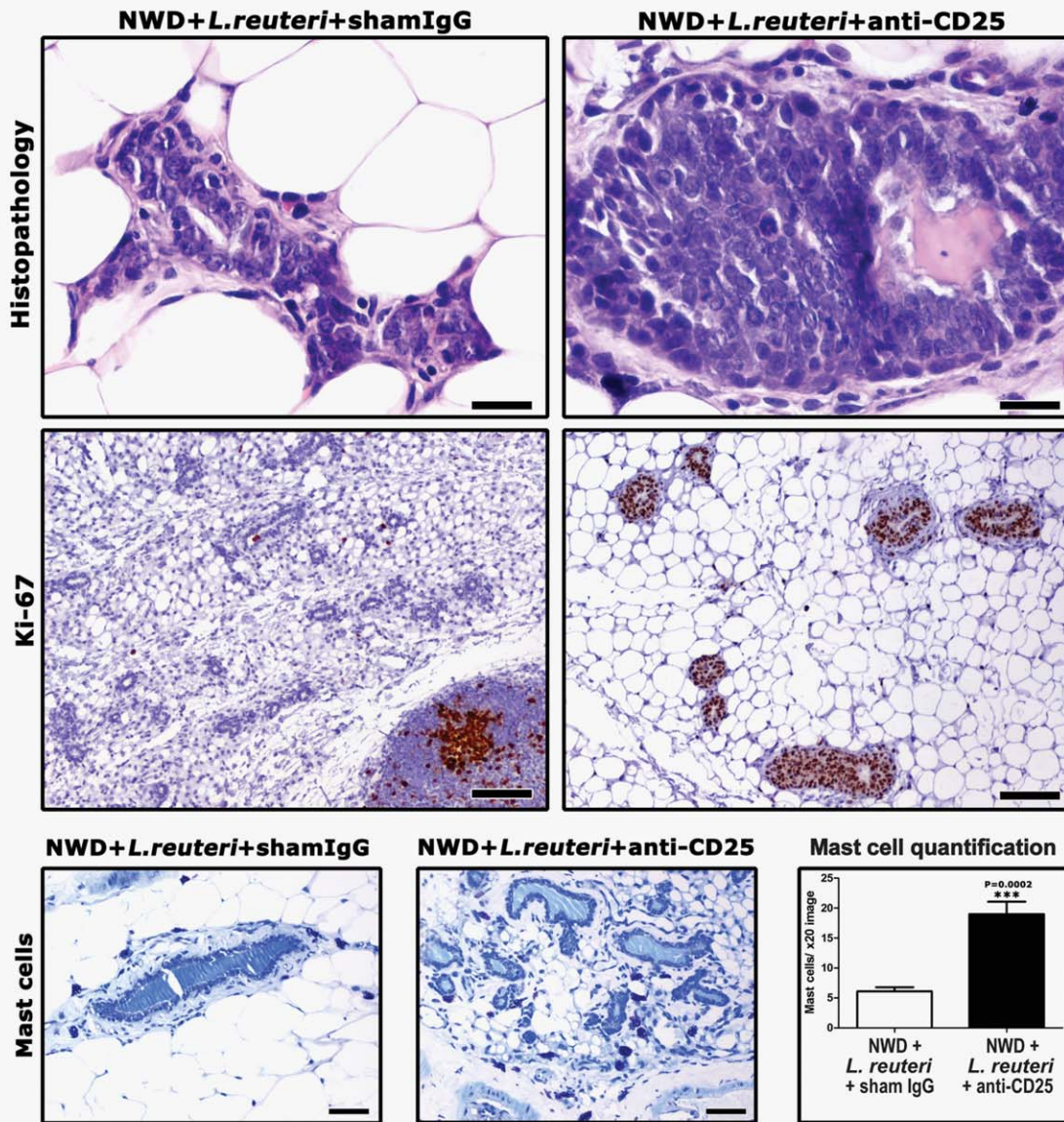


Figure 4. The protective effect of *L. reuteri* depends upon CD25⁺ cells. The depletion of CD25⁺ cells negated the protective effect offered by *L. reuteri* in mice consuming the western-type diet. Compare the mammary gland from a CD25 cell-depleted mouse with carcinoma *in situ* with the mammary gland from a sham antibody-treated mouse showing only low grade hyperplasia without atypia. The mammary epithelium of sham antibody-treated control mice had occasional Ki-67⁺ proliferating cells (Ki-67⁺ cells in the lymphoid follicle germinal center of the mammary lymph node serves as a stain internal positive control). In contrast, CD25 cell-depleted mice had a hyperproliferative mammary gland epithelium. Mammary gland pathology after CD25⁺ cell depletion co-existed with a significant increase of mast cells. Histopathology: Hematoxylin and eosin. Ki-67: DAB chromogen, Hematoxylin counterstain. Mast cells: Toluidine Blue stain. Bars: Histopathology = 25 μ m; Ki-67 = 100 μ m; Mast cells = 50 μ m.

epithelium. Utilizing NF κ -B p65- and c-Jun-specific immunohistochemistry and morphometric counts of positively-stained cells in comparable (non-necrotic) tumor areas, we found that *L. reuteri* induced a significant decrease of nuclear NF κ -B ($p < 0.0002$) and of c-Jun ($p < 0.0025$) in neoplastic cells (Fig. 6). Interestingly, tumor cells neighboring to necrotic areas in tumors from *L. reuteri*-fed mice had increased c-Jun expression (Supporting Information Fig. S6). These data, when combined, suggested that consuming *L.*

reuteri served to upregulate apoptosis in the tumor prone mammary tissue.

***L. reuteri*-treated donor CD4⁺CD45RB^{lo}CD25⁺ immune cells imbued transplantable anti-cancer protection into HER2 transgenic recipient mice**

To identify a cellular mechanism of the benefits of the probiotic, and having previously shown a protective role for CD25⁺ cells in Swiss mice, we purified CD4⁺CD45RB^{lo}CD25⁺ cells

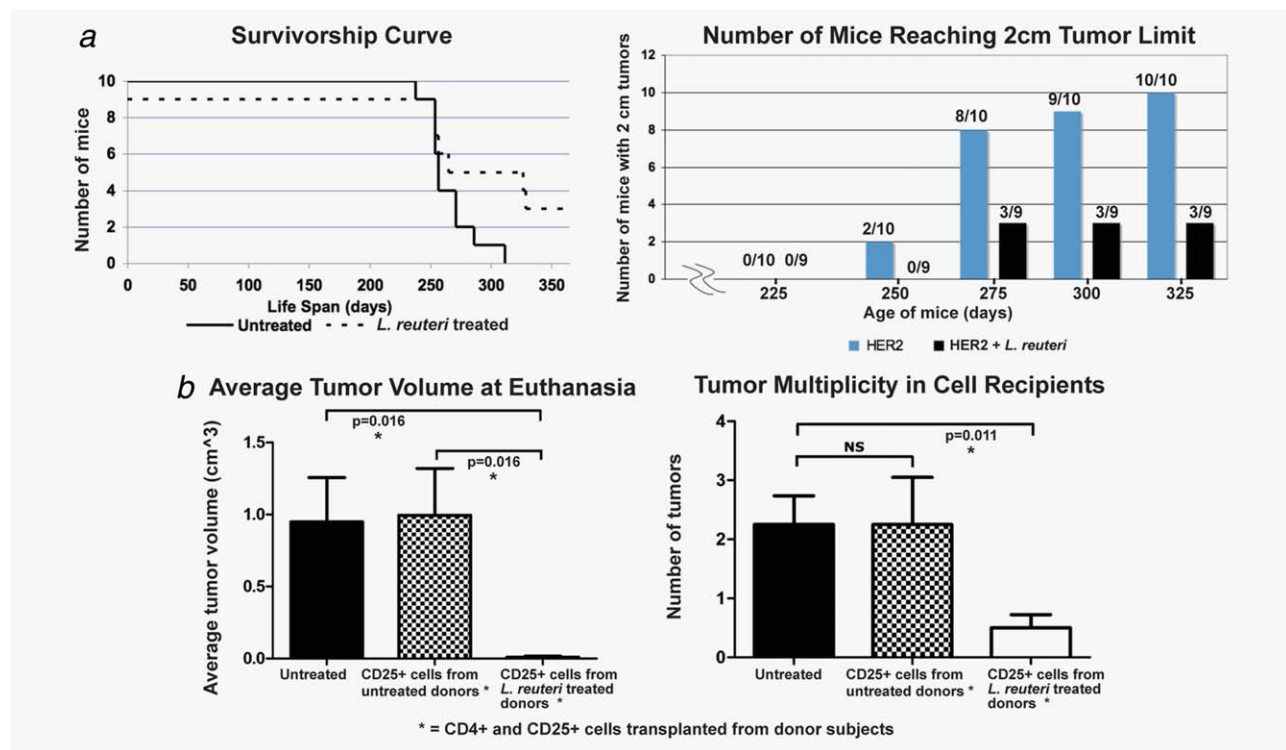


Figure 5. Tumor-free survival was increased in HER2 transgenic mice after eating purified *L. reuteri*. Mice exhibited a delayed onset of mammary tumor burden (a). This anti-cancer protection effect was transplantable using highly purified CD4⁺CD45RB^{lo}CD25⁺ immune cells into HER2 transgenic recipient mice (b).

from syngeneic FVB wild-type mice that were either untreated or supplemented with oral administration of *L. reuteri* in the drinking water, as above. Afterward, 3×10^5 purified CD4⁺CD45RB^{lo}CD25⁺ lymphocytes were injected intraperitoneally into randomly untreated recipient HER2/neu mutant animals with some pre-existing tumor burden at 6 months of age. All recipient animals were euthanized at 3–4 weeks after adoptive cell transfer. We found that transfer of *L. reuteri* treated CD4⁺CD45RB^{lo}CD25⁺ cells alone was sufficient to inhibit or suppress mammary tumors when compared with untreated animals or with animals that received cells from untreated FVB donor mice. Mice that received *L. reuteri* treated CD4⁺CD45RB^{lo}CD25⁺ cells had a significantly ($p < 0.016$) smaller tumor burden and fewer tumors ($p < 0.011$) compared with the other treatment groups (Fig. 5b). Taken together, this was in line with earlier work showing that intestinal bacteria impact systemic carcinogenic events *via* recruitment of bacteria-triggered CD4⁺CD25⁺ immune cells,^{13,30} and supported our hypothesis that lactic acid organisms such as *L. reuteri* may protect from cancer by favorably biasing the immune system through induction of anti-inflammatory CD4⁺CD25⁺ regulatory T cells.

Discussion

Here we used two etiologically divergent animal models to investigate mechanisms of lactic acid microbes previously shown to impact breast cancer outcomes in women.^{22,23} In

both mouse models we found that exposure to *L. reuteri* in their drinking water ultimately lowered risk of mammary carcinogenesis in an immune cell-dependent manner. Importantly, we show that (i) lower cancer risk outcomes were achievable in both models using purified *L. reuteri* organisms alone, regardless of the baseline diet, (ii) an environmental microbial exposure such as *L. reuteri* was sufficient to inhibit carcinogenesis, even with a pre-existing genetic predilection to cancer, and (iii) *L. reuteri* cancer-protective outcomes required microbe-triggered CD25⁺ cells. We concluded that microbes such as *L. reuteri* may ultimately offer a tractable public health approach to foster protective immunity and help counteract the accumulated dietary and genetic carcinogenic events integral in the Westernized diet and lifestyle.

Outbred Swiss mice consuming a Westernized “fast food” (NWD) chow mimicked adiposity patterns and associated breast cancer risk in people,^{1–6} with lower risk of carcinogenesis after consuming *L. reuteri*. For an outbred strain, the Swiss mice had a remarkably increased diet-associated risk to develop pre-neoplastic and neoplastic mammary lesions in the present study. This risk may not exclusively be related to the diet. Although control-diet-fed Swiss mice had significantly less hyperplasia from westernized-diet fed mice, several mice of the control group did have some degree of focal or multifocal mammary gland hyperplasia. These changes, although mild, are rather unusual based on our empiric observations in virgin mice of other strains. This matches

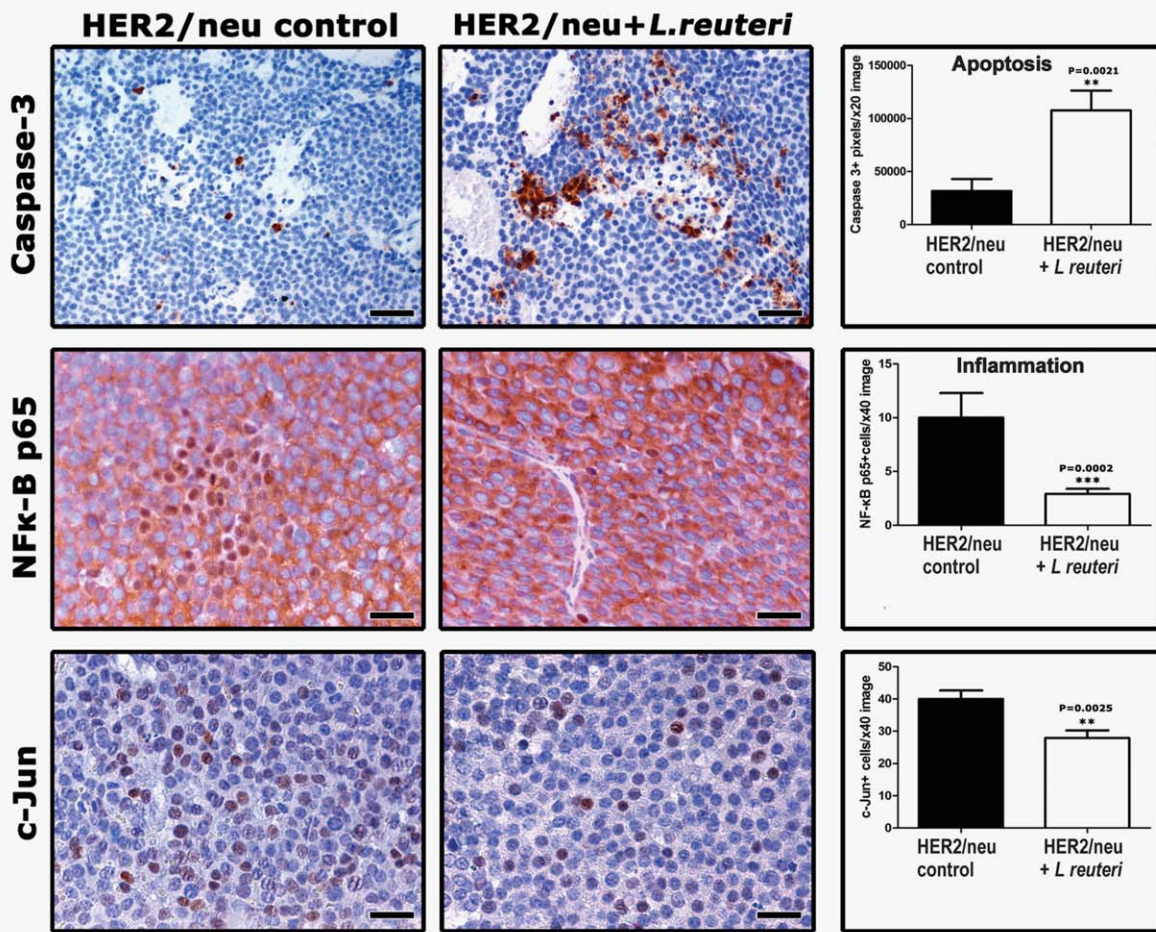


Figure 6. Feeding of *L. reuteri* increases apoptosis and suppresses anti-apoptotic signals in HER2/neu mammary tumors. Morphometric counts of immunohistochemically labeled tumors suggest that *L. reuteri* consumption associates with a significant increase in neoplastic cell apoptosis (Caspase-3+ cells) and a significantly decrease in NFκ-B- and c-Jun-derived tumor cell-originating anti-apoptotic signals. DAB chromogen, Hematoxylin counterstain. Bars: Caspase-3 = 50 μm; NFκ-B p65 and c-Jun = 25 μm.

previously reported observations in mammary tissue of untreated FVB mice, an inbred strain closely related to the white Swiss mouse.^{31,32} Unlike FVB mice, the aged outbred Swiss mouse we examined showed no spontaneous mammary tumors when fed with a normal diet. Nonetheless, the possibility that mouse strain-related factors may contribute to some extent in the westernized diet-induced mammary tumorigenesis we report here cannot be excluded.

Hyperproliferative and preneoplastic mammary glands in NWD-fed mice were surrounded by particularly increased numbers of mast cells. This is highly suggestive for a cross-talk between these cells and the proliferating epithelium, and reminiscent of their accumulation in the expanding terminal end bud of the mammary ductal system at puberty.³³ Mast cells facilitate abnormal gland growth by extracellular matrix degradation and the remodeling of adjacent tissues. They also produce growth factors and cytokines. Studies in both humans and mice describe significant contributions of mast cells into tumor evolution and development.^{33–35}

Mast cell accumulations were inversely related to the *L. reuteri*-induced restoration of the Foxp3⁺ cell population within mammary lymph nodes. In addition, mast cells were robustly increased after depletion of CD25⁺ cells. This result suggests that gut bacteria-triggered CD25⁺Foxp3⁺ Treg cells can not only inhibit systemic mast cell-associated pathologies, such as allergy and autoimmune reactions,²⁹ but also early stage carcinogenesis, as well. Consequently, it supports the concept that exposures to lactic acid microbiota stimulate Treg cell-orchestrated immune networks capable of inhibiting inflammatory diseases, including early stage malignant transformation.^{35,36}

These observations associated with a Western diet in aging Swiss female mice support a mechanistic model whereby lactic acid microbes impact host immunity, which in turn affects obesity and carcinogenesis that subsequently alters host immunity, and so forth. Once initiated, these processes may become self-sustaining. We postulate that oral dosing of *L. reuteri* imparts immune homeostasis that then

maintains systemic immune balance relying upon sustained immune tolerance. Diet and microbe-induced failure of tolerance unifies these data with prior work involving inflammation, obesity, and cancer.^{18,37} Diverse disorders such as asthma and autoimmune diseases associated with Westernized living are widely believed to result from insufficient immune tolerance that is essential for sustained systemic health. Westernized diets are also low in vitamin D, a nutrient that normally works together with IL-10 to enforce immune tolerance and protect against inflammatory disorders³⁸ and some types of cancer.³⁹

To overcome diet and obesity-associated biases in understanding carcinogenesis, we separately tested roles of *L. reuteri* and candidate immune cells in slender erbB2 mutant mice, with a genetic predisposition to mammary tumors mimicking 30% of breast cancers diagnosed in women.¹⁵ Our finding that mammary tumors from HER2/neu mice consuming *L. reuteri* have increased intratumoral necrosis and apoptosis is similar to our previous findings in *ApcMin*⁺ mice after adoptive transfer of microbe-induced Treg cells. Regressive intestinal adenomas in these *ApcMin*⁺ mice after Treg cell transfer were characterized by umbilicated necrotic centers and increased tumor cell apoptosis.³⁷ NFκ-B and c-Jun expression of tumor cells have both been associated with increased tumor malignancy in humans^{40,41} and mouse models of mammary cancer, including the HER2/neu mice.⁴² An important aspect of the tumor-promoting effect of these pleiotropic proteins lies in their tumor cell survival and anti-apoptotic effects.⁴³ We found that dietary *L. reuteri* blocked NFκ-B-p65 nuclear translocation and c-jun expression in mammary tumor cells. This effect may have rendered HER2/neu tumor cells less resistant to apoptosis, and thus explain, at least in part, the probiotic microbe-induced anti-neoplastic effect.

It is not entirely clear how host hormones triggered by microbial symbionts such as *L. reuteri* may impact the carcinogenic processes. We have recently discovered that consumption of *L. reuteri* up-regulates plasma levels of the neuropeptide hormone oxytocin^{17,18,44} that also benefits damaged tissue repairs,⁴⁴ a concept that may extrapolate from

wound healing to breast cancer.⁴⁵ It has also been shown that oxytocin may counteract some of the immune suppressive effects of estrogen.⁴⁶ These observations, if translatable to humans, may be particularly important given the proposed roles of oxytocin in protection from breast cancer.⁴⁷ In addition, emerging data relate oxytocin with metabolism and the immune system^{46,48} connecting microbial symbionts such as *L. reuteri* with immune fitness in evolutionary success.⁴⁶

Our previous work and that of many others has revealed that CD4⁺CD25⁺ cells are a basic cellular carrier of gut microbially triggered signals that shape the immune system to restore homeostasis in inflammatory-associated pathologies, cancer, and reproductive health.^{13,49,50} We discovered that the *L. reuteri* benefit was transferable to other recipient animals using CD4⁺CD45RB^{lo}CD25⁺ cells alone, as long as those cells were from immune-competent donor animals. In our previous studies we have shown that homeostatic properties reside predominantly in this sub-population of Treg cells.¹⁴ In the present study transplanted CD4⁺CD45RB^{lo}CD25⁺ immune cells from *L. reuteri*-primed donor animals were sufficient to completely recapitulate the *L. reuteri*-induced phenomenon in HER2 transgenic recipient host animals. In conclusion, we found that ingested microbes impact the systemic immune system at the whole organism level and serve to counteract a genetic predisposition to cancer, even within the tumor micro-environment. Beneficial microbes may ultimately offer tractable population-based approaches to target cancer.

Author Contributions

Conceived and designed the experiments: JRL TP EJA SEE. Performed the experiments: JRL TP TL BJV YMI AC SM SEE. Analyzed the data: JRL TP EJA SEE. Contributed reagents/materials/analysis tools: JRL, TP. Wrote the article: JRL TP EJA SEE.

Acknowledgements

The authors thank James Versalovic for the gift of ATCC-PTA-6475 *Lactobacillus reuteri*, and special thanks to James G. Fox for encouragement and support.

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