Inhibitory effects of *Bifidobacterium*-fermented soy milk on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced rat mammary carcinogenesis, with a partial contribution of its component isoflavones

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High consumption of soya bean and soya bean-related products is hypothesized to contribute to protection against breast cancer. Soya bean is a rich source of genistein, a putative cancer chemopreventive agent. Fermented soya milk (FSM), which is made of soya milk fermented with the *Bifidobacterium breve* strain Yakult, contains larger amounts of the isoflavone aglycones genistein and daidzein than unfermented soya milk. In the present study, we examined the effects of FSM and its component isoflavone mixture (genistein:daidzein 4:1) on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced mammary carcinogenesis in rats. Starting at 7 weeks of age, female Sprague–Dawley rats were given PhIP at a dose of 85 mg/kg body wt by intragastric administration four times a week for 2 weeks. They were fed control high fat basal diet or experimental high fat diet containing 10% FSM or 0.02 or 0.04% isoflavone mixture during and after carcinogen exposure. The incidences (percentage of rats with tumors) of mammary gland tumors were 71% in the control diet group, 51% in the FSM group and 68 and 61% in the groups treated with isoflavone mixture at 0.02 and 0.04%, respectively. Mammary tumor multiplicities (number of tumors per rat) were 1.2 ± 0.2 for 10% FSM, 2.2 ± 0.4 for 0.02% isoflavone mixture and 1.5 ± 0.3 for 0.04% isoflavone mixture, being clearly smaller than the control diet value (2.6 ± 0.5). Furthermore, feeding of FSM and the isoflavone mixture at both doses reduced the sizes of mammary tumors. Since the amounts of isoflavones in 10% FSM are approximately equivalent to those in the 0.02% isoflavone mixture, the chemopreventive activity of FSM could be partly attributable to the presence of isoflavones such as genistein and daidzein.

**Introduction**

Breast cancer is one of the most common cancers in women. It accounts for almost 30% of all newly diagnosed malignant neoplasms in the USA (1). Several epidemiological studies have demonstrated that a high consumption of meat is associated with an increased risk of breast cancer (2,3). Moreover, the risk of breast cancer was found to be significantly elevated with an increased intake of well-done to very well-done meat (4). On the other hand, Gertig et al. reported that there was no association between meat intake or the cooking method of meat and the risk of breast cancer (5). Of the series of carcinogenic heterocyclic amines (HCAs) that have been identified in cooked meat, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), being the most abundant HCA in cooked foods (6), induces cancers in the mammary glands of female rats at a high incidence (7–9). Therefore, PhIP may play an important role in the development of breast cancer in humans.

High consumption of soya bean and soya bean-related products has been suggested to contribute to a reduction in the risk of breast cancer in epidemiological studies (10). The average daily consumption of soya bean and its products per person in Japan is much higher than the value for Americans (11,12). Many components, such as isoflavones, protease inhibitors, saponins and inositol hexaphosphate, have been investigated in the search for candidates responsible for the chemopreventive effects (13). Among these, isoflavones, particularly genistein, have been demonstrated to show several kinds of biological activity (14). Genistein inhibits protein tyrosine kinase and topoisomerase II activities (15,16). Genistein also has an inhibitory effect on angiogenesis (17), antioxidative potential (18) and phytoestrogenic activity (19). Among four isoflavones tested (genistein and daidzein and their β-glucoside conjugates, genistin and daidzin), genistein was reported to most effectively inhibit the growth of LNCaP, a human prostate cancer cell line, while daidzein showed a weak inhibitory effect (20). The β-glucoside conjugates genistin and daidzin exerted far less influence. Genistein can be absorbed in the upper small intestine (21), whereas the β-glucoside conjugate genistin needs conversion to an aglycone through the action of a β-glucosidase produced by intestinal bacteria before being absorbed.

In general, the amounts of genistein and daidzein are much less than genistin and daidzin in soya bean and its related products, such as soy milk and tofu (12). Based on biological activities of the isoflavone aglycones and glucoside conjugates and the conversion efficiency of glucoside conjugates to aglycones *in vivo*, soya bean-related products containing higher amounts of aglycones than glucoside conjugates of isoflavones would be preferable for cancer prevention. Glucoside conjugates may be converted to aglycones by microbes during fermentation. Therefore, we produced fermented soya milk (FSM) with *Bifidobacterium breve* strain Yakult in an attempt to increase the amounts of isoflavone aglycones and also to improve the flavor of soya milk. Using the FSM thus obtained, inhibitory effects on PhIP-induced mammary tumor development in Sprague–Dawley female rats were examined. To determine the active principles in FSM, the effect of an isoflavone mixture of genistein and daidzein was also tested.

**Abbreviations:** FSM, fermented soya milk; HCAs, heterocyclic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.
The concentrations of components in diets containing test samples were 18 weeks and 57% at 20 weeks, with a...glucose from co-existent glucoside-conjugated forms. The soybean isoavone mixture...and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological examination and pathological diagnosis of mammary tumors was made according to criteria as described previously (25).

**Statistical analysis**

The significance of differences in incidences of tumors was analyzed by the $\chi^2$ test. Other data were examined using Welch’s $t$-test. A $P$ value of $<0.05$ was regarded as significant.

**Results**

Daily dietary intake in the PhIP-treated groups was 21.3 g/day with the control diet, 20.7 g with 10% FSM, 20.9 g with the 0.02% isoflavone mixture and 22.6 g with the 0.04% isoflavone mixture, on average (Table II). Administration of FSM and isoflavone mixture did not affect the feeding and behavior of rats. The body weights of PhIP-treated rats ranged from 325.3 to 344.6 g and those of vehicle-treated rats were 353.9–362.3 g (Table II). Body weight gain was thus slightly decreased by PhIP treatment, but no significant differences were observed among the PhIP-treated groups. Also, no differences were observed regarding liver weight in each group (Table II). During the study, five rats in the PhIP-treated groups and one rat in the vehicle-treated group died of acute problems due to intubation, and these were not included in the effective numbers.

The results of sequential observation of mammary gland tumors by palpation in the PhIP-treated groups are shown in Figure 1. Palpable tumors in the control diet group first appeared at 12 weeks after the first dosing with PhIP and the incidences (percentage of rats with tumors) reached 55% at 18 weeks and 57% at 20 weeks, with a final multiplicity (no. of tumors/rat) of 1.0 ± 0.2 per rat. Incidences and multiplicities of palpable tumors at 20 weeks were 31% and 0.5 ± 0.1 for the 10% FSM group, 41% and 0.9 ± 0.2 for the 0.02% isoflavone mixture group and 41% and 0.9 ± 0.2 for the 0.04% isoflavone mixture group. Thus, both incidences and multiplicities were lower in the experimental diet groups as compared with the control diet group. All animals were killed at 20 weeks after the first dosing with PhIP. Additional non-palpable tumors were also detected at termination.

### Table I. Composition of the diets used in the present study

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g/kg diet)</th>
<th>Basal diet</th>
<th>10% FSM</th>
<th>Isoflavone mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.02%</td>
</tr>
<tr>
<td>Casein</td>
<td>235.0</td>
<td>191.2</td>
<td>235.0</td>
<td>235.0</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Corn starch</td>
<td>95.0</td>
<td>95.0</td>
<td>95.0</td>
<td>95.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>317.0</td>
<td>284.6</td>
<td>317.0</td>
<td>317.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>59.0</td>
<td>59.0</td>
<td>58.8</td>
<td>58.6</td>
</tr>
<tr>
<td>Corn oil</td>
<td>235.2</td>
<td>211.4</td>
<td>235.2</td>
<td>235.2</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>41.1</td>
<td>41.1</td>
<td>41.1</td>
<td>41.1</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>11.8</td>
<td>11.8</td>
<td>11.8</td>
<td>11.8</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>FSM</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoflavone mixture</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>0.0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>

### Materials and methods

**Chemicals**

PhIP-HCl was obtained from the Nard Institute Ltd (Osaka, Japan). Flavone, used as an internal standard for the analysis of isoflavones, was obtained from Tokyo Kasei Co. (Tokyo, Japan). Genistein and daidzein, which were also applied as standard substances to estimate isoflavone contents, were purchased from Sigma Chemical Co. (St Louis, MO) and Seikagaku Corp. (Tokyo, Japan), respectively.

**Preparation of FSM and isoflavone mixture**

A seed culture of *Bifidobacterium breve* strain Yakult was added to soy milk (Shikokukakouki Co. Ltd, Tokushima, Japan) at 10 ml/l and fermentation was allowed to proceed statically at 37°C for 18 h under anaerobic conditions. The pH and viable cell counts of the FSM were 4.6 and 4.1, respectively. The FSM obtained was then lyophilized and levels of genistein, genistin, daidzein and daidzin in FSM were analyzed according to the method described by Kikuchi-Hayakawa et al. (22). These amounts were 1318 µg for genistein, 26 µg for genistin, 677 µg for daidzein and 269 µg for daidzin per gram lyophilized material. The crude protein, crude fat and ash content levels in the lyophilized FSM were 44, 24 and 32%, respectively.

The soybean isoflavone mixture was prepared as follows. First, defatted soybeans were extracted with boiled water and the extracted material was applied to a Sepabeads SP207 column (Mitsubishi Kasei Co., Tokyo, Japan) and eluted with methanol. After evaporation the residue was redissolved in 50% (v/v) ethanol and hydrolyzed with 5% (v/v) sulfuric acid to remove glucose from co-existing glucoside-conjugated forms. The soybean isoflavone mixture consisted of ~80% genistein; most of the remainder was daidzein.

**Mammary carcinogenesis experiments in rats**

Female Sprague–Dawley rats, 6 weeks old, were purchased from CLEA Japan Inc. (Tokyo, Japan) and housed 3 rats/cage with wood chips in an air-conditioned animal room with a 12 h light/dark cycle. Starting at 7 weeks of age, a total of 204 rats were fed a modified AIN-76A high fat basal diet (33.5% corn oil; CLEA Japan Inc.) and an experimental diet containing 0.02 or 0.04% of the isoflavone mixture or 10% FSM during and after carcinogen exposure. Doses of the isoflavone mixture and FSM were chosen on the basis of the chemopreventive data shown in our previous report (23). The compositions of the diets used in the present study are shown in Table I. The concentrations of components in diets containing test samples were isocalorically adjusted to those in the basal diet. Diets were stored at 4°C until use. A fresh diet was provided to the rats once a week. Dietary isoflavones were confirmed to be stable under these conditions by HPLC analysis.

All animals except those for vehicle treatment received intragastric intubations of PhIP at a dose of 85 mg/kg body wt four times weekly for 2 weeks, as described previously (24). Body weight and diet intake were measured weekly and animals were monitored daily for their general health and mammary tumor development. At 20 weeks after the first dosing with PhIP, all animals were killed under ether anesthesia and the numbers and sizes of all mammary tumors were recorded. The length ($L$), width ($W$) and height ($H$) of each lesion were measured with calipers and tumor volumes were calculated using the formula $V = L \times W \times H \times \pi / 6$. Tumor samples and organs demonstrating apparent abnormalities were fixed with 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological examination and pathological diagnosis of mammary tumors was made according to criteria as described previously (25).
and experimental diet groups were diagnosed histologically as can be calculated to be 0.2 mg/day/kg body wt (12). These

Mean number of rats with mammary tumors per effective number of rats. due to the possible presence of several other anticarcinogens

PhIP-treated in rats (26).

Incidences, multiplicities and volumes of mammary tumors mammary carcinogenesis. Consistent with these data, soybeans, Treatment Incidence Multiplicity Volume

Table II. Diet intake in each group and body and liver weights at termination

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Diet intake (g/day/rat)</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Relative liver weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>42</td>
<td>21.3 ± 1.1</td>
<td>344.6 ± 8.6</td>
<td>11.3 ± 0.3</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>10% FSM</td>
<td>39</td>
<td>20.7 ± 1.1</td>
<td>341.7 ± 6.7</td>
<td>10.2 ± 0.2</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>0.02% isoflavones</td>
<td>41</td>
<td>20.9 ± 1.6</td>
<td>330.3 ± 6.1</td>
<td>10.3 ± 0.3</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>0.04% isoflavones</td>
<td>41</td>
<td>22.6 ± 1.5</td>
<td>325.3 ± 5.9</td>
<td>10.8 ± 0.2</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>Vehicle-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>12</td>
<td>21.6 ± 2.1</td>
<td>362.3 ± 7.0</td>
<td>11.1 ± 0.2</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>10% FSM</td>
<td>11</td>
<td>19.4 ± 1.7</td>
<td>365.9 ± 7.0</td>
<td>11.0 ± 0.2</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>0.04% isoflavones</td>
<td>12</td>
<td>19.7 ± 0.3</td>
<td>353.9 ± 7.4</td>
<td>10.9 ± 0.2</td>
<td>3.1 ± 0.3</td>
</tr>
</tbody>
</table>

aMean ± SE.

Table III. Incidences, multiplicities and volumes of mammary tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence (no. of tumors/rat)</th>
<th>Multiplicity (no. of tumors/rat)</th>
<th>Volume (cm²/rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>30/42 (71)</td>
<td>2.6 ± 0.5b</td>
<td>4.1 ± 1.3</td>
</tr>
<tr>
<td>10% FSM</td>
<td>20/39 (51)</td>
<td>1.2 ± 0.2c</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>0.02% isoflavones</td>
<td>28/41 (68)</td>
<td>2.2 ± 0.4</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>0.04% isoflavones</td>
<td>25/41 (61)</td>
<td>1.5 ± 0.3a</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>Vehicle-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>0/12 (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10% FSM</td>
<td>0/11 (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.04% isoflavones</td>
<td>0/12 (0)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

aNumber of rats with mammary tumors per effective number of rats.

bSignificantly different (P < 0.05) from the PhIP + control diet value by Welch’s t-test.

cSignificantly different (P < 0.05) from the PhIP + control diet value by Welch’s t-test.

dSignificantly different (P < 0.05) from the PhIP + control diet value by Welch’s t-test.

Discussion

In the present study, the fermented soy product FSM was found to inhibit the development of tumors in the mammary glands of PhIP-treated Sprague–Dawley female rats. In addition, the isoflavone components genistein and daidzein also showed chemopreventive action against PhIP-induced mammary carcinogenesis. Consistent with these data, soybeans, soy protein isolate and miso, produced from soybeans by fermentation, have been reported to show inhibitory effects on chemically and irradiation induced mammary carcinogenesis in rats (26–29).

Quantification analysis revealed FSM to contain 1318 and 677 µg/g genistein and daidzein, respectively, in lyophilized material. This indicates that the isoflavone contents in 10% FSM are 132 p.p.m. for genistein and 68 p.p.m. for daidzein, being almost equivalent to the 0.02% isoflavone mixture in the diet. FSM caused a greater reduction of mammary tumor development than the 0.02% isoflavone mixture; this might be due to the possible presence of several other anticarcinogens such as protease inhibitors, phytosterol, saponins and inositol hexaphosphate, which could act additively or synergistically with isoflavones. The average daily consumption of soybean and their products per person in Japan in 1997 was 68.9 g, being 4.8% of total food intake (30). On the basis of these data, the total intake of genistein and genistin by the Japanese can be calculated to be 0.2 mg/day/kg body wt (12). These levels are almost 60-fold less than those of genistein taken from the FSM diet in rats.

It has been reported that administration of genistein prenatally and/or neonatally inhibits dimethylbenz[a]antra-
Carcinogenesis


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References


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