Effect of Probiotics, *Bifidobacterium breve* and *Lactobacillus casei*, on Bisphenol A Exposure in Rats

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Bisphenol A (BPA), a putative endocrine disruptor, may be taken up by humans via the diet and have adverse effects on human health. In this study, we evaluated whether the probiotics, *Bifidobacterium breve* strain Yakult (BbY) and *Lactobacillus casei* strain Shirota (LcS), could exert a protective effect against dietary exposure to BPA. A group of rats fed on a diet containing 5% BbY or 5% LcS showed three advantageous effects compared to the control group; (i) the area under the blood concentration-time curve of BPA after its oral administration was significantly decreased, (ii) the amount of BPA excreted in the feces was significantly greater (2.4 times), and (iii) the percentage of BPA bound to the sediment fraction of the feces was significantly higher. These results suggest that BbY and LcS reduced the intestinal absorption by facilitating the excretion of BPA, and that these probiotics may suppress the adverse effects of BPA on human health.

Key words: bisphenol A; probiotic; bifidobacteria; lactic acid bacteria; rat

Environmental estrogens are chemicals that bind to estrogen receptors, mimic estrogenic actions, and may have adverse effects on human health. 1, 2) One such compound is bisphenol A (BPA), a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins that is used extensively in the food packaging industry and dentistry. 3, 4) For example, treatment of pregnant CF-1 mice with BPA for 7 d at a dose that is within the range of typical environmental exposure significantly reduced the number of days between vaginal opening and first vaginal estrus in their female offspring that were located between two female litter mates in the womb. 5) Ikezuki et al. 6) have reported the accumulation of BPA in early fetuses and considerable exposure during the prenatal period in humans. Sakamoto et al. 7) have recently shown enterohepatic circulation of BPA and suggested that the adverse effects of BPA were enhanced by repeated exposure. Although BPA was absorbed from the small intestine, most was conjugated with glucuronide, and only 2–8% of free BPA was retained in the circulating blood. 8) However, BPA-glucuronide does not exhibit estrogenic activity, 9) so free BPA contributes to the harmful actions.

Bifidobacteria and lactic acid bacteria are used in the production of dairy products. These bacteria are also becoming popular with consumers due to their beneficial effects on human health. It has been reported that bifidobacteria and lactic acid bacteria have many beneficial functions such as anti-microbial effects against pathogenic microorganisms, 10, 11) modulation of the immune system, 12–14) anti-tumor activity, 15, 16) and anti-oxidative effects. 17, 18) Bifidobacteria and lactic acid bacteria also have the ability to bind food carcinogens such as heterocyclic amines, 19, 20) benzo[a]pyrene and aflatoxin B1. 19) Furthermore, the administration of freeze-dried viable *Lactobacillus casei* has suppressed the urinary mutagenicity arising from ingesting fried ground beef in humans by binding to heterocyclic amines. 21) This evidence led to the expectation that bifidobacteria and lactic acid bacteria would bind to BPA in the gastrointestinal tract and might be effective in protecting humans from the adverse effects of this compound by preventing its intestinal absorption.

We investigated in this study the effects of feeding the probiotics, *Bifidobacterium breve* strain Yakult (BbY) and *Lactobacillus casei* strain Shirota (LcS), on the fecal excretion and blood concentration of BPA in rats.

**Materials and Methods**

**Chemicals.** BPA (GC grade > 99%) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan), and BPA-d16 (98 atom % D) was purchased from Cambridge...
Isotope Laboratories (MA, USA). N\textsubscript{2}O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce Biotechnology (IL, USA). The pesticide residue-grade methanol, n-hexane, dichloromethane, and acetone were obtained from Wako Pure Chemical Industries (Osaka, Japan).

**Bacteria.** BbY and LcS were obtained from the Culture Collection Research Laboratory of Yakult Central Institute for Microbiological Research (Tokyo, Japan). The bacteria were cultured in modified GAM broth (Nissui Pharmaceutical Co., Tokyo, Japan) containing 1% glucose and 0.1% Tween 80 for 18 h at 37°C. Following washing in a 20 mM potassium phosphate buffer (pH 7.0), the bacterial cells were harvested by centrifugation at 8,500 \times g for 15 min. To prepare heat-killed cells, the viable cells were suspended in distilled water and incubated at 80°C for 30 min. The suspension was then centrifuged at 3,000 \times g for 20 min to collect a cell pellet. This cell pellet was washed three times with distilled water and used as heat-killed cells. For the animal experiments, viable cells of BbY and LcS were lyophilized, powdered, and mixed with an AIN-76 diet.

**Animals.** Nine-week-old female Fischer 344 rats were purchased from Clea Japan (Tokyo, Japan), and were individually housed in metal cages. After acclimatization for 1 week, the rats were randomly divided into three groups for each experiment, the average body weight not being significantly different among the groups. The rats had free access to food and water, and were housed in a vivarium with controlled temperature (25°C), humidity (55%) and light (12-h light/dark cycle) throughout the experimental period. Food consumption and body weight were recorded every 2 d. The Ethics Committee for Animal Experiments at Yakult Central Institute approved all animal procedures.

**Test for the relationship between the amount of BPA excreted in feces and the oral dose of BPA.** Twenty-four rats were divided into three groups each containing eight rats and were fed an AIN-76 diet (Table 1, control group) for 4 d. After fasting for 16 h, rats weighing 118–138 g were given 10 g of food containing 0.1 mg of BPA (0.8 ± 0.0 mg/kg) and were fed within the next 24 h. The rats were then fed with the AIN-76 diet or AIN-76 diet containing bacteria for 6 d. All the feces from each rat were collected and lyophilized.

**Test for the lowering effect of feeding bacteria on the elevation of BPA level in the blood.** The rats were divided into groups each containing six rats and fed with the following diets: AIN-76 diet (Table 1) for the control group; AIN-76 diet containing lyophilized BbY (5.0%, w/w) for the BbY 5.0% group; AIN-76 diet containing lyophilized LcS (5.0%, w/w) for the LcS 5.0% group. The rats were fed on the diets for 4 d. After fasting for 16 h, the rats weighing 118–138 g were given 10 g of food containing 0.1 mg of BPA (0.8 ± 0.0 mg/kg) and were fed within the next 24 h. The rats were then fed with the AIN-76 diet or AIN-76 diet containing bacteria for 6 d. All the feces from each rat were collected and lyophilized.

**BPA extraction from the feces and blood.** A methanol solution of BPA-d\textsubscript{16} (1 \mu g in 0.1 ml) was added to lyophilized feces (0.5 g) or hemolysed blood (0.3 ml). BPA and BPA-d\textsubscript{16} were extracted twice with methanol (10 ml). The extracts were dried by evaporation under nitrogen gas, and then the residue was suspended in 5 ml of methanol. Distilled water (0.125 ml) was added to the solution which was washed twice with n-hexane (2.5 ml). The methanol layer was dried by evaporation under N\textsubscript{2} and then the residue was suspended in 1 ml of methanol. The solution was adjusted to pH 3 by adding 0.125 N HCl, and then 3.75% NaCl (4 ml) was added. BPA and BPA-d\textsubscript{16} were extracted twice with dichloromethane (5 ml), the extracts being dried with anhydrous sodium sulfate (1.5 g). The solvent was removed by evaporating under N\textsubscript{2}, and then the residue was suspended in 0.2 ml of dichloromethane. The dichloro-

<table>
<thead>
<tr>
<th>Component</th>
<th>Control group</th>
<th>Bacteria feeding group</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>2.5%</td>
</tr>
<tr>
<td>Casein</td>
<td>12.5</td>
<td>12.1</td>
</tr>
<tr>
<td>d-Methionine</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>(\alpha)-Corn starch</td>
<td>15.0</td>
<td>14.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>42.5</td>
<td>41.3</td>
</tr>
<tr>
<td>Cellulose fiber</td>
<td>5.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
<td>4.9</td>
</tr>
<tr>
<td>AIN-76 mineral mix</td>
<td>3.5</td>
<td>3.4</td>
</tr>
<tr>
<td>AIN-76 vitamin mix</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Skim milk</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Freeze-dried bacteria</td>
<td>0.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>
methane suspension was subjected to solid-phase separation by passing through Sep-Pak Plus (silica) cartridges (Waters Co., MA, USA) and then eluted with acetone. The eluate (10 ml) was dried by evaporation under N₂, and then the residue was suspended in dichloromethane (0.26 ml) and BSTFA (0.04 ml). The mixture was left at room temperature for 1 h, and a 1-μl aliquot was used for GC-MS analysis.

**Determination of BPA in the aqueous and sediment fractions of the feces of rats fed on the control diet, 5.0% BbY- or 5.0% LcS-containing diet.** Lyophilized feces (0.5 g) were suspended in 2 ml of a 20 mM potassium phosphate buffer (pH 7.0). After this suspension had been centrifuged at 1,200 × g for 15 min, the supernatant (0.95 ml) was collected. An ethanol solution of BPA-d₁₆ (0.1 mg in 0.05 ml) was added to the supernatant (0.95 ml). BPA and BPA-d₁₆ were extracted with dichloromethane (1.0 ml). The extract (0.2 ml) was dried by evaporating under nitrogen, and then the residue was suspended in dichloromethane (0.16 ml) and BSTFA (0.04 ml). An aliquot of 1 μl of the sample was used for a GC-MS analysis. BPA in the sediment fraction was calculated by subtracting BPA in the supernatant from total BPA.

**Determination of BPA sequestered to bacteria.** To determine the amount of BPA sequestered to bacteria, twenty milligrams of viable cells or heat-killed cells was suspended in 3.8 ml of a 20 mM potassium phosphate buffer (pH 7.0). An ethanol solution of BPA (0.04 mg in 0.2 ml) was added to each suspension, and the mixture was incubated at 37°C for 1 h. After incubating, the mixture was centrifuged at 1,200 × g for 15 min, and the supernatant (0.95 ml) was collected. Extraction and quantification of BPA in the supernatant was followed by the determination of BPA in the aqueous fraction of the feces. Three samples for each strain were measured. BPA sequestered to bacteria was calculated by subtracting BPA in the supernatant from total BPA added to the suspension.

**GC-MS analysis.** GC-MS was carried out with a GC17A instrument (Shimadzu, Kyoto, Japan) equipped with an HP-5 column (30 m × 0.25 mm I.D., 0.25 μm film thickness; Agilent, DE, USA) coupled to a QP5000 mass spectrometer (Shimadzu, Kyoto, Japan). The mass spectrometer was operated in the electron impact mode at 70 eV, and in the ion-monitoring mode at 357 (TMSBPA) and 368 (TMS-BPA-d₁₆) m/z. Mass resolution, detector voltage, and sampling rate were evaluated at 1,400, 1.5 kV, and 0.2 s, respectively. Helium 2 was used as the carrier gas at a flow rate of 1.5 ml/min. The oven temperature was maintained at 60°C for 1 min and then increased to 280°C at a rate of 10°C/min (maintained for 5 min). Each sample (1 μl) was injected into the GC which was operated in the splitless mode with an injector port temperature of 280°C. TMS-BPA and TMS-BPA-d₁₆ were analyzed by GC-MS in the selected ion monitoring (SIM) mode.

**Statistics.** Data obtained from the animal experiments were analyzed by using one-way ANOVA and Dunnett’s multiple test and are expressed as the mean and standard deviation (S.D.). With respect to the in vitro study, the mean and S.D. of each result from three samples are shown.

**Results**

**Experimental administration dose and duration of BPA**

To determine the appropriate administration dose and duration, BPA was orally administered at doses of 0.1 mg, 0.25 mg, and 0.5 mg per rat (Fig. 1). BPA was detected in the feces of all groups from the same day of administration. The total amount of BPA excreted in the feces appeared to level off 7 d after the oral administration (Fig. 1). As shown in Fig. 2, the total amount of BPA excreted in the feces 7 d after the oral administration increased proportionally with the dosage (R = 0.92). When BPA was orally administered at a dose of 0.05 mg per rat, BPA was detected in the feces, but under the quantifiable limit. We chose 0.1 mg/rat, the minimum dosage within the proportional relationship between oral dosage and total excretion in the feces, as the dose of BPA, and 7 d as the collection period for rat feces for the next experiments.

**Effects of feeding BbY and LcS on the fecal excretion of BPA by rats**

Figure 3 shows the effect of feeding BbY and LcS on BPA excretion of BPA by the rats. In the control group, 17% of orally administered BPA was excreted in the feces 7 d. On the other hand, the total amount of BPA excreted in the feces of the BbY 5.0% group and LcS 5.0% group was 2.4 times and 2.5 times higher than that of the control group, respectively (P < 0.01). Although no significant difference was detected, the total amount of BPA excreted in the feces of the BbY 2.5% group and LcS 2.5% group was 1.5 times and 1.8 times higher than that of control group, respectively. Both the total weight of feces from the rats and the concentration of BPA in the feces from the rats also increased dose-dependently of BbY and LcS, and significant differences were detected in both the BbY 5.0% group and LcS 5.0% group. No difference was apparent in the food consumption and body weight gain between the control group and bacteria-fed groups (Table 2).

**BPA sequestered to fecal sediment and bacteria**

Table 3 shows BPA in the aqueous and sediment fraction of feces from rats fed on the control diet, and 5.0% BbY- and 5.0% LcS-containing diets. Feeding the diet containing 5.0% BbY or 5.0% LcS did not change BPA in the aqueous fraction, but that in the sediment
fraction was significantly increased.

BPA sequestered to bacterial cells was examined by incubating the bacterial cells with BPA. The bacterial cells were removed by centrifugation, and BPA in the supernatant was measured. Viable BbY and LcS sequestered 0.87 mg and 0.58 mg of BPA per mg of cells, respectively. BPA sequestered to these bacteria was not decreased by a heat treatment.

Effect of BbY and LcS on the elevation of BPA level in the blood
Since feeding the diet containing 5.0% BbY or 5.0% LcS significantly enhanced the fecal excretion of BPA, we further investigated the effects of BbY and LcS on the elevation of BPA level in the blood. As shown in Fig. 4, the blood level of BPA in the control rats reached a maximum (0.7 μM) 0.5 h after a single oral administration of BPA, and decreased time dependently. In the rats fed on a diet containing 5.0% BbY, the BPA level in the blood was significantly lower than that of the control group after 0.5 h (P < 0.01) and 2 h (P < 0.05). In the case of the diet containing 5.0% LcS, the BPA level after 2 h was lower than that of the control diet. The BPA area under the curve between 0 and 6 h after the oral administration for the rats fed on a diet containing 5.0% BbY (0.9 ± 0.1 μM×h, P < 0.01) and 5.0% LcS (0.9 ± 0.3 μM×h, P < 0.05) was significantly lower than that of the rats fed on the control diet (1.7 ± 0.9 μM×h).
Table 2. Body Weight Gain and Food Consumption of the Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight gain (g/7 d)</th>
<th>Food consumption (g/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.1 ± 4.4</td>
<td>10.7 ± 0.6</td>
</tr>
<tr>
<td>BbY 2.5%</td>
<td>12.8 ± 3.9</td>
<td>11.0 ± 1.0</td>
</tr>
<tr>
<td>BbY 5.0%</td>
<td>12.4 ± 0.9</td>
<td>10.6 ± 0.8</td>
</tr>
<tr>
<td>LcS 2.5%</td>
<td>13.3 ± 9.8</td>
<td>11.1 ± 0.7</td>
</tr>
<tr>
<td>LcS 5.0%</td>
<td>13.6 ± 6.1</td>
<td>10.3 ± 1.5</td>
</tr>
</tbody>
</table>

N = 8  
BbY, Bifidobacterium breve strain Yakult  
LcS, Lactobacillus casei strain Shirota

Table 3. BPA in the Aqueous Fraction and Sediment Fraction of a Fecal Suspension from Rats Fed on a Control Diet, and a 5.0% BbY- or 5.0% LcS-Containing Diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Aqueous fraction</th>
<th>Sediment fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.4 ± 0.3</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>BbY 5.0%</td>
<td>0.4 ± 0.1</td>
<td>3.6 ± 1.3**</td>
</tr>
<tr>
<td>LcS 5.0%</td>
<td>0.3 ± 0.1</td>
<td>4.0 ± 1.4**</td>
</tr>
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</table>

**P < 0.01 vs. control (Dunnett’s multiple test). N = 8.  
BPA, bisphenol A  
BbY, Bifidobacterium breve strain Yakult  
LcS, Lactobacillus casei strain Shirota
Discussion

As BPA can easily leach out from industrial products such as plastic tableware and the lacquer applied as a food can lining,\textsuperscript{22} humans may be routinely exposed to this compound via their diet. BPA is absorbed from the small intestine and enters the enterohepatic circulation, prolonging its presence in the body.\textsuperscript{7,23} BPA is mainly metabolized to its glucuronide conjugate by glucuronyl transferases during its passage through the intestinal wall, but a portion of BPA that escapes the intestinal conjugation enters the circulating blood in its free form.\textsuperscript{8} Since free BPA, unlike BPA-glucuronide, exhibits estrogenic activity,\textsuperscript{9} for decreasing the level of free BPA in the circulating blood is effective in protecting against exposure to this putative environmental disruptor. It was revealed in this study that feeding a diet containing 5.0% BbY or 5.0% LcS halved the area under the blood concentration-time curve of BPA in rats after a single oral administration of this compound.

The amount of BPA excreted in the feces of rats fed on a diet containing BbY or LcS was significantly higher than that of rats fed on the control diet. Therefore, the decrease in the blood concentration of BPA appears to be attributable to the acceleration of its fecal excretion.

The total amount of feces from rats fed on a diet containing BbY or LcS was significantly higher than that from rats fed on the control diet. Olivares \textit{et al.}\textsuperscript{24} have reported that the oral administration of probiotic strains to humans led to an increase in stool volume by increasing the water content of the feces, possibly through the production of short-chain fatty acids.

Although the total amount of feces was increased by feeding BbY and LcS, the concentration of BPA in the feces was not diluted, but rather was concentrated when compared with the control group. In addition, BPA retained in the sediment fraction of feces from rats fed on a diet containing BbY or LcS was significantly higher than that of rats fed on the control diet. Moreover, viable cells of BbY and LcS sequestered BPA in the intestine and promoted the excretion of this compound into the feces. Since heat-killed bacterial cell pellets of BbY and LcS also sequestered BPA, this sequestration was not due to the energy-dependent uptake. Endo \textit{et al.} have reported that the lactic acid bacteria, \textit{Lactococcus} strains, could adsorb BPA, and this ability could have been due to the hydrophobic binding effect.\textsuperscript{25} Hydrophobic binding is therefore one possible mechanism to account for sequestration of BPA to the cells of BbY and LcS in this study. Since it has been reported that probiotic strains of \textit{L. rhamnosus} bound aflatoxin B1, and that hydrophobic interaction played a major role in this binding,\textsuperscript{26} there is a possibility that BbY and LcS could bind not only BPA but also to other hydrophobic compounds.

As already mentioned, \textit{Lactococcus} strains could bind to BPA in vitro,\textsuperscript{25} although their in vivo effects have never been investigated. In the present study, we have shown that the blood concentration of BPA after its oral administration was decreased by feeding with lactic acid bacteria through the acceleration of its fecal excretion. These findings suggest that feeding BbY and LcS reduced the risk of exposure to endocrine disruptors.
Further investigation is needed to clarify whether feeding these lactic acid bacteria could lead to reducing the endocrine disrupting effect of BPA.

In conclusion, it is suggested that BbY and LcS might be useful for reducing the adverse effects promoting BPA excretion into the feces. Therefore, BbY and LcS might be useful for reducing the adverse effects of BPA.

Acknowledgments

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References