Suppressive Effects of Bifidobacteria on Lipid Peroxidation in the Colonic Mucosa of Iron-Overloaded Mice

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ABSTRACT

The antioxidative effects of live bifidobacteria on lipid peroxidation in the colonic mucosa were investigated. *Bifidobacterium bifidum* strain Yakult, which has been used for production of fermented milk, most effectively inhibited lipid peroxidation catalyzed by ferrous iron in liposomes among 10 species of bifidobacteria from human intestinal flora. Oral administration of *B. bifidum* strain Yakult for 2 wk significantly decreased the level of lipid peroxide (thiobarbituric acid reactive substance) in the colonic mucosa of iron-overload mice (Fe 0.07% in diet). The iron concentrations in plasma and cecum contents were not affected by administration of *B. bifidum* strain Yakult. *Bifidobacterium bifidum* strain Yakult had no chelating or incorporating activity for ferrous iron in vitro. Therefore, the antioxidative effect of *B. bifidum* strain Yakult in the colonic mucosa was not thought to be based on the removal of ferrous iron from the reaction system of lipid peroxidation. These results suggested that *B. bifidum* strain Yakult protected the colonic mucosa from oxidative injury without inhibiting iron absorption.

(Key words: colon, lipid peroxide, iron, bifidobacteria)

Abbreviation key: ADA = N-(2-acetamido)imidodiacetic acid, IC$_{50}$ = 50% inhibitory concentration, Nitroso-PSAP = 2-nitroso-5-(N-propyl-N-sulfopropylamino)phenol, TBARS = thiobarbituric acid reactive substance.

INTRODUCTION

Iron is essential for maintaining proper cell functions (Boldt, 1999). However, iron, especially ferrous iron, may cause deleterious reactions. Ferrous iron is a catalyst in the Haber-Weiss reaction, hydroxyl radical formation, and is involved in initiation and propagation of lipid peroxidation. Therefore, the reactivity of iron is usually tightly controlled by binding to transport and storage proteins. Because the above-described control mechanism on the activity of iron does not work in the intestinal tract, the colonic mucosa that usually contacts with colon contents may be exposed to a high risk of oxidative injury (Babbs, 1990).

Some epidemiological and animal studies have suggested high iron intakes may produce oxidative stress in the colonic mucosa and enhance the risk of colon cancer and ulcerative colitis (Kawai et al., 1992; Knekt et al., 1994; Nelson, 1992; Siegers et al., 1988; Wurzelmann et al., 1996). Younes et al. (1990) reported that the amount of lipid peroxide in the colonic tissue of mice was increased dependent on the iron contents of diets. In a chemically induced colorectal cancer model in rats, intake of high levels of dietary iron increased the risk of colon cancer compared with intake of the minimum daily requirement (35 mg/kg of diet). Babbs (1990, 1992) hypothesized that high levels of unab sorbed fecal iron resulting from excessive dietary iron may catalyze the production of oxygen radicals, thus amplifying mucosal injury in ulcerative colitis and increasing the incidence of colon cancer. The suppression of oxidative stress in the colon would be effective for the prevention of these diseases.

It has been reported that dietary phytate (myo-inositol hexaphosphate) canceled the effect of iron overload ing on lipid peroxidation in the colonic mucosa and the colon cancer risk (Porres et al., 1999; Nelson et al., 1992). Phytate, which chelates iron in a form that does not support the Haber-Weiss reaction, on the other hand, is a well-known inhibitor of iron absorption (Hallback et al., 1989). Decreasing iron absorption in people such as children or menstruants who are liable to be deficient in iron is undesirable. Therefore, a food material to protect the colonic mucosa from oxidative stress without iron chelation is needed.

Intestinal bacteria contact with the colonic mucosa and may affect the redox status in the mucosa. To find an antioxidative material that protects the colonic mucosa from oxidative stress, we have investigated the bacteria that exist in fermented milk and may contact with the colonic mucosa after oral administration. Bifidobacteria are one of the major bacteria in human intestinal flora and are used to produce fermented milk.
They have many beneficial functions, such as control of gastrointestinal infections (Tanaka, 1995), stimulation of the immune system (Yasui et al., 1989), anti-ulcer activity (Nagaoka et al., 1994; Yamamoto et al. 1994) and anti-tumor activity (Sekine et al., 1995). However, data relating to the antioxidative effect of the bacteria on colonic mucosa have not been available.

There have been some reports concerning animal experimental systems that increased lipid peroxide in the colonic mucosa by iron overloading. Nevertheless, the relationship between the overloading duration and the contents of lipid peroxide has not been reported. In this study, therefore, we examined the relationship, and then verified the effect of oral administration of bifidobacteria on lipid peroxidation in the colonic mucosa. As a result, we found that Bifidobacterium bifidum strain Yakult, which has been used to produce fermented milk, had an antioxidative effect on the colonic mucosa.

**MATERIALS AND METHODS**

**Bacteria**

Bifidobacterium adolescentis YIT 4011, Bifidobacterium angulatum YIT 4012, Bifidobacterium bifidum strain Yakult, B. bifidum YIT 4039, Bifidobacterium breve YIT 4014, B. breve strain Yakult, Bifidobacterium catenulatum YIT 4016, Bifidobacterium dentium YIT 4017, Bifidobacterium gallicum YIT 4085, Bifidobacterium infantis YIT 4018, Bifidobacterium longum YIT 4021, and Bifidobacterium pseudocatenulatum YIT 4072 were obtained from the Culture Collection Research Laboratory of the Yakult Central Institute for Microbiological Research (Tokyo, Japan). These strains were obtained from human intestines (feces). Bifidobacterium bifidum strain Yakult and B. breve strain Yakult have been used to produce fermented milks.

**Culture and Treatment of Bacterial Cells**

The bifidobacteria were cultured in modified GAM broth (Nissui Seiyaku Co., Tokyo, Japan) containing 1% glucose anaerobically using Aneropack Kenki (Mitsubishi Gas Chemical Co., Tokyo, Japan) for 18 h at 37°C. The bacterial cells were harvested by centrifugation at 8500 × g for 15 min, following saline washes. For animal experiments, B. bifidum strain Yakult was lyophilized and powdered.

The concentrations of bacteria were expressed by viable bacterial counts (cfu/ml), which were calculated from colony counts on modified GAM agar plates (Nissui Seiyaku Co.) after anaerobic incubation at 37°C for 2 d.

When the metabolic system of the bacteria had to be inactivated, bacterial cells were autoclaved at 105°C for 15 min. The autoclaved cells did not form colonies after anaerobic incubation at 37°C for 2 d.

**Inhibitory Activity on Lipid Peroxidation in Liposomes**

To determine the inhibitory ability of bifidobacteria against lipid peroxidation in the cell membrane, we used liposome as a substrate for peroxidation. L-α-phosphatidylcholine (0.1 g; type XV-E; from egg yolk; Sigma Chemical Co., St. Louis, MO) was dissolved in diethyl ether (10 ml), and distilled water (0.6 ml) was added. The mixture was sonicated with an ultrasonic disrupter CA-44882 (Kaijo Co., Tokyo, Japan), while it was evaporated to dryness under vacuum at 0°C. Thirty milliliters of 0.1 M N-(2-acetamido)iminodiacetic acid (ADA) sodium buffer (pH 6.7) was added, and the mixture was sonicated for 15 min at 0°C. The suspension was centrifuged at 1500 × g for 10 min at 4°C, and supernatant was used as a liposomal suspension.

The liposomal suspensions were incubated with 0.1 M ADA-sodium buffer (pH 6.7), 48 μM ferrous chloride, and 1 mM sodium ascorbate for 2 h at 37°C in the presence or absence of the bifidobacterium or phytic acid (dodecasodium salt; Sigma Chemical Co.). A part of the mixture was incubated in the presence of 0.16% glucose. After the incubation, the lipid peroxide in the mixture was quantified as thiobarbituric acid reactive substance (TBARS) by the method of Kosugi et al. (1991) in the presence of EDTA and measurement of fluorescence intensity, as follows. In a test tube, 0.1 ml of test sample (the liposomal mixture after the incubation), 0.2 ml of 8.1% sodium deoxycholate, 1.5 ml of 20% acetic acid solution adjusted at pH 3.5 with 10 N NaOH, 0.05 ml of 0.8% butylated hydroxytoluene in acetic acid, 1.0 ml of 0.8% thiobarbituric acid, 0.7 ml of 5 mM EDTA and 0.5 ml of distilled water were placed, in that order. This mixture was kept at 5°C for 60 min and then heated at 100°C for 60 min. TBARS was extracted with 5.0 ml of n-butanol-pyridine (15:1) and quantified from the fluorescence intensity (excitation: 515 nm, emission: 553 nm, Hitachi 650-10S fluorescence spectrophotometer, Hitachi, Ltd., Tokyo, Japan). When the mixture contained glucose, the amount of lipid peroxide was estimated as follows: The residual glucose in the mixture was quantified using F-kit glucose (Boehringer Mannheim, Mannheim, Germany). Glucose solutions at the same concentrations as the residual glucose levels were treated by the method of Kosugi et al. (1991) in the presence of EDTA and fluorescence intensity was measured. The amount of lipid peroxide was estimated as TBARS by subtracting the levels of TBARS derived from glucose from those of TBARS in the mixture.
Inhibition rates for lipid peroxidation were calculated according to the following equation:

\[
\text{Inhibition rate for lipid peroxidation} = \frac{(C - T)}{(C - B)} \times 100.
\]

C: TBARS of a sample incubated without the bacterial cells or phytate. T: TBARS of a sample incubated with the bacterial cells or phytate. B: TBARS of distilled water.

Each sample was tested at four or five dilution levels. Fifty percent inhibitory concentration (IC_{50}) values were calculated from inhibition curves.

### Decreasing Rate of “Free” Ferrous Iron

A reaction mixture consisting of 0.1 M ADA-sodium buffer (pH 6.8), 35 mM sodium metabisulfite, and 48 \(\mu\)M ferrous chloride was incubated with or without washed bacterial cells (2.0 \(\times\) 10^6 cfu/ml) or phytate (3.5 mM) at 37°C for 60 min. After the incubation, the mixture was centrifuged at 8500 \(\times\) g for 15 min and the supernatant was recovered. One ml of the supernatant was mixed with 25 \(\mu\)l of 2-nitroso-5-(N-propyl-N-sulfopropylamino)phenol (Nitroso-PSAP). Fifteen minutes later, absorbance at 750 nm was measured. A decreasing rate of “free” ferrous iron in the mixture was calculated according to the following equation:

\[
\text{Decreasing rate of “free” ferrous iron} = \frac{(A750(C) - A750(S))}{A750(C)} \times 100.
\]

A750(S): Absorbance at 750 nm (Sample: incubation with cell or phytate). A750(C): Absorbance at 750 nm (Control: incubation without cell or phytate).

### Animals and Diets

Six-week-old male BALB/cA mice were obtained from Clea, Ltd., Tokyo, Japan. After acclimation for 1 wk, the mice were randomly divided into groups as described below.

Animal experiment 1 (to investigate the relationship between the contents of lipid peroxide in the colonic mucosa and the duration of iron overload): The mice were divided into two groups each containing eight mice and fed with AIN-76 diet or the iron-enriched diet (Table 1) for 1, 2, or 4 weeks.

Animal experiment 2 (to verify the suppressive effect on lipid peroxidation of \(B.\ bifidum\) strain Yakult in the colonic mucosa): The mice were divided into four groups each containing 12 mice and fed with the following diets; iron-enriched (control) diet (Table 1) for a control group; iron-enriched diet containing the lyophilized \(B.\ bifidum\) strain Yakult (0.4%) for a B 0.4% group; iron-enriched diet containing the lyophilized \(B.\ bifidum\) strain Yakult (2.0%) for a B 2.0% group; and AIN-76 (American Institute of Nutrition, 1977) diet (Table 1) for a normal diet group. The diet treatment was continued for 2 wk.

The mice had free access to food and water and were accommodated in a room with controlled temperature (25°C), humidity (55%), and light (12-h light/dark cycle) during the whole experimental period. Food consumptions and BW were recorded every 2 d and every week, respectively.

After the dietary treatment, the mice were killed. They were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL), and blood was obtained via the abdominal vein and mixed with heparin. The colon and cecum were removed with the contents.

All animal procedures were approved by the Ethical Committee for the Animal Experiments of Yakult Central Institute.

### Preparation of Plasma and Colonic Mucosa and Treatments of Cecum Contents

Plasma was obtained from heparinized blood by centrifugation at 1500 \(\times\) g for 15 min. The colon was incised and rinsed with saline. The colonic mucosa was scraped with a clean glass microscope slide and homogenized in 1.15% KCl. The cecum was incised, and the contents were recovered. The cecum contents were dispersed in saline (sample: saline = 1 volume : 10 volume) and centrifuged at 13,000 \(\times\) g for 15 min. Supernatant was recovered to measure soluble iron (total and ferrous iron) concentrations.

### Biochemical Analysis

Mucosal lipid peroxide was measured using the TBARS assay in the presence of EDTA. The mucosal homogenate was treated by the method of Kosugi et al. (1991), and fluorescence intensity was measured (excitation: 515 nm, emission: 553 nm), which was the same way with in vitro assay. Protein concentration was measured with the BCA protein assay kit (Pierce Co., USA).
Figure 1. Relationship between the amounts of thiobarbituric acid reactive substance (TBARS) in the colonic mucosa and duration of iron overload. ○ TBARS of iron-overloaded mice (mice fed with the iron-enriched diet); ▲ TBARS of mice in the normal diet group (mice fed with the AIN-76 diet); ––, means; MDA, malondialdehyde. Results of two-way ANOVA: Diet, \( P < 0.05 \); Term, \( P < 0.05 \); and Diet \( \times \) Term; \( P < 0.05 \).

Iron concentrations in plasma and cecum contents were measured by a colorimetric method with a commercial kit (Fe-C test Wako, Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Ferrous iron concentration was measured with Fe-C test Wako without a reductant.

Results of two-way ANOVA: Diet, \( P < 0.05 \); Term, \( P < 0.05 \); and Diet \( \times \) Term; \( P < 0.05 \).

Statistics

Data obtained from the animal experiment were analyzed using two-way (animal experiment 1; the data presented in Figure 1) or one-way (animal experiment 2; the data presented in Figure 2 and Table 2) ANOVA to determine the significance \( (P < 0.05) \). When the result of ANOVA was significant, non-paired t-test (animal experiment 1) or Tukey’s multiple comparison test (animal experiment 2) was conducted. The VisualStat statistical software (ver. 4.5J; StatSoft Inc., Tulsa, USA) was employed.

RESULTS

Inhibitory Activity on Lipid Peroxidation in Liposomes

The inhibitory activity of the bifidobacteria from human intestinal flora on lipid peroxidation in liposomes was examined (Table 3). Production of lipid peroxide (TBARS) was decreased dependently on bacterial concentrations. The \( I_{C50} \) of washed cells of the tested strains were \( 2.0 \times 10^8 \) cfu/ml \( (\log_{10} 8.3) \) to \( 4.0 \times 10^9 \) cfu/ml \( (\log_{10} 9.6) \), and the highest inhibitory activity was demonstrated by \( B. bifidum \) strain Yakult. The activity of \( B. bifidum \) strain Yakult was not affected by incubation with glucose to activate energy metabolism or autoclave treatment before the incubation to inactivate metabolism.

Relationship Between the Contents of Lipid Peroxide in Colonic Mucosa and the Duration of Iron Overload

Figure 1 shows the relationship between the contents of lipid peroxide in the colonic mucosa and the duration of iron overload. The TBARS in the colonic mucosa increased significantly in the mice fed with the iron-enriched diet containing \( 0.07\% \) iron beginning 1 wk after the start of administration as to the AIN-76 diet (control group) \( (P < 0.05) \). The TBARS of the colonic mucosa was increased dependently on the duration of iron overloading \( (P < 0.05) \). On the day 1 wk after the start of diet treatment, the TBARS levels in the colonic mucosa of a part of the iron-overloaded mice were almost equal to those of the control mice. On the day 2 wk after the start of diet treatment, the TBARS levels of all the mice fed with the iron-enriched diet were higher than those for the control group. Thus, we chose 2 wk as iron overloading duration for the experiment, which verified the suppressive effects of bifidobacteria on lipid peroxidation in the colonic mucosa.

Suppressive Effects on Lipid Peroxidation in Colonic Mucosa

Figure 2 shows the effect of oral administration of \( B. bifidum \) strain Yakult on lipid peroxidation in the co-
Table 2. Change in iron concentrations in plasma and cecum contents, feed consumption, and BW. 1

<table>
<thead>
<tr>
<th></th>
<th>Normal2</th>
<th>Control3</th>
<th>B 0.4%4</th>
<th>B 2.0%5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contents in the cecum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total soluble iron (mg/dl)</td>
<td>0.85 ± 0.21b</td>
<td>5.57 ± 3.75a</td>
<td>5.58 ± 4.83b</td>
<td>4.66 ± 2.26a</td>
</tr>
<tr>
<td>Ferrous iron (mg/dl)</td>
<td>0.41 ± 0.11b</td>
<td>1.21 ± 0.67a</td>
<td>1.20 ± 0.67a</td>
<td>0.96 ± 0.44ab</td>
</tr>
<tr>
<td>Plasma iron concentration (µg/dl)</td>
<td>323 ± 67b</td>
<td>351 ± 43a</td>
<td>334 ± 52a</td>
<td>347 ± 79a</td>
</tr>
<tr>
<td>Average feed consumption (g/head per day)</td>
<td>3.6</td>
<td>3.7</td>
<td>3.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Body weight at the start of the test (g)</td>
<td>25.5 ± 1.4a</td>
<td>25.5 ± 1.1a</td>
<td>25.4 ± 1.0a</td>
<td>25.4 ± 0.9a</td>
</tr>
<tr>
<td>Body weight at the end of the test (g)</td>
<td>29.6 ± 1.6a</td>
<td>29.2 ± 1.9a</td>
<td>28.3 ± 1.4a</td>
<td>28.0 ± 1.5a</td>
</tr>
</tbody>
</table>

1Values except average feed consumption represent the mean ± standard deviation. Values in the same line with different superscript letters shows significant difference (P < 0.05).
2Mice fed with the AIN-76 diet.
3Mice fed with the iron-enriched (control) diet.
4Mice fed with the 0.4% Bifidobacterium bifidum strain Yakult containing iron-enriched diet.
5Mice fed with the 2.0% B. bifidum strain Yakult containing iron-enriched diet.

The mucosal TBARS of iron-overloaded mice was increased compared with the mice fed with the normal diet (P < 0.05). TBARS of the mice fed with the diet containing B. bifidum strain Yakult (0.4 or 2.0%) decreased significantly compared with the mice fed with the iron-enriched diet containing no B. bifidum strain Yakult (P < 0.05). There was no significant difference in total soluble iron or ferrous iron concentration in the cecum content, except for the normal diet group (Table 3). No difference was observed in plasma iron concentration or BW among the four groups.

Decreasing Rate of “Free” Ferrous Iron

Decreasing rates of “free” ferrous iron in the reaction mixtures, when incubated with B. bifidum strain Yakult or dodecasodium phytate at the concentrations of IC50 for lipid peroxidation (2.0 × 10⁸ cfu/ml and 3.5 mM, respectively), were determined. The data in Table 4 show that B. bifidum strain Yakult did not decrease the concentration of “free” ferrous iron in the reaction mixture, while phytate decreased it by about 30%.

**DISCUSSION**

In the present study, we used a dietary iron (ferrous fumarate) to induce oxidative stress in the colonic mucosa. Although the dose (0.07% Fe in diet) we used was lower than that of Younes et al. (1990) and almost equal to that of Porres et al. (1999), and the iron overloading duration was shorter than the 4 wk that was previously reported, a significant increase in mucosal TBARS was observed (Figure 1). The increase in TBARS was dependent on the duration of iron overload. We then investigated the effect of oral administration of B. bifidum.
Lipid peroxidation was accelerated by a catalytic activity of ferrous iron. Dietary phytate, which chelates ferrous iron to generate a form that does not support the Haber-Weiss reaction, inhibited lipid peroxidation in the colonic mucosa (Porres et al., 1999). Kot et al. (1995) reported bifidobacteria had an iron incorporating (accumulating) activity. Therefore, the antioxidative mechanism may include an iron chelating to generate a form that dose not support the Haber-Weiss reaction or incorporation of iron into bacterial cells. However, B. bifidum strain Yakult did not decrease the concentration of “free” ferrous iron in vitro (Table 2). The soluble iron concentrations of the cecum contents were not different among the groups, except the normal diet-feeding group (Table 4). Therefore, we did not think that the antioxidative effect of B. bifidum strain Yakult in the colonic mucosa was based on removal (chelating, incorporation in bacterial cells, or oxidation) of ferrous iron from a reaction system of lipid peroxidation. To clarify the mechanism of the antioxidative effect, further investigations need to be conducted.

Phytate is a potent inhibitor of iron absorption due to a chelating activity (Hallberg et al., 1989; Pallauf et al., 1999). Bifidobacterium bifidum strain Yakult that did not chelate iron may protect the colonic mucosa from oxidative injury without inhibiting iron absorption.

Various synthetic and natural antioxidants for foods have been reported. However, few were reported to act in the colonic mucosa except phytate. Oxidative stress in the colonic mucosa is presumably involved in the pathogenesis of inflammatory bowel diseases and colon cancer (Babbs, 1990, 1992; Kawai et al., 1992; Knekt et al., 1994; Nelson, 1992; Siegers et al., 1988; Wurzelmann et al., 1996). The results of this study suggest B. bifidum strain Yakult protects the colonic mucosa against oxidative stress, and then, may decrease the incidence of inflammatory bowel diseases and colon cancer.

**CONCLUSIONS**

Bifidobacterium bifidum strain Yakult most effectively inhibited lipid peroxidation catalyzed by ferrous iron in liposomes among 10 species of bifidobacteria from human intestinal flora and did not decrease the concentration of “free” ferrous iron in vitro. Oral administration of B. bifidum strain Yakult decreased the levels of lipid peroxide in the colonic mucosa of iron overload mice. The iron concentrations of cecum contents were not affected by administration of B. bifidum strain Yakult. These results did not mean that the antioxidative effect of B. bifidum strain Yakult in the colonic mucosa was based on removal of ferrous iron from the

**Table 4. Decreasing rate of “free” ferrous iron.**

<table>
<thead>
<tr>
<th>Bifidobacterium bifidum strain Yakult</th>
<th>Decreasing rate of “free” ferrous iron</th>
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<tbody>
<tr>
<td>Phytic acid (dodecasodium salt)²</td>
<td>27.2</td>
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</table>

¹Sample concentration was equal to IC₅₀ of the lipid peroxidation in liposome (2.0 × 10⁸ cfu/ml).

²Sample concentration was equal to IC₅₀ of the lipid peroxidation in liposome (3.5 mM).
reaction system of lipid peroxidation. This is different from the case of phytate that is an inhibitor of iron absorption. Bifidobacterium bifidum strain Yakult may protect the colonic mucosa from oxidative injury without inhibiting iron absorption.

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REFERENCES


