Preventive Effect of *Lactobacillus delbrueckii* subsp. *bulgaricus* on the Oxidation of LDL

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*Lactobacillus delbrueckii* subsp. *bulgaricus* 2038 was examined for its activity to prevent the oxidation of the erythrocyte membrane *in vitro*, and the oxidation of LDL *in vivo*.

Strain 2038 produced radical scavengers that reacted with 1,1-diphenyl-2-picrylhydrazl (DPPH) during cultivation. Moreover, the ethereal extract from the supernatant of the culture prevented the oxidation of the erythrocyte membrane *in vitro*.

As an *in vivo* study, male F344 rats were fed on diets containing 20% fresh soybean oil (or 13% oxidized oil and 7% fresh oil) with 10% freeze-dried powder of the 2038 culture (or with skim milk powder) for 4 weeks. The level of thiobarbituric acid-reactive substances was lower in the low-density lipoproteins (per milligram of cholesterol) from rats fed on the oxidized oil with freeze-dried powder of the 2038 culture than without it. The level of vitamin E in the plasma was higher in the rats fed on the oxidized oil with the freeze-dried powder than without it.

Key words: 1,1-diphenyl-2-picrylhydrazl (DPPH); erythrocyte membrane; thiobarbituric acid-reactive substances (TBARS); malondialdehyde (MDA); vitamin E

The incidence of atherosclerotic heart disease is increased in patients with hypercholesterolemia.11 Regulation of the serum cholesterol level is important to prevent atherosclerosis, and it has been shown that atherosclerosis could be suppressed by controlling the level of cholesterol in the serum.29 The hypcholesterolemic effects of lactic acid bacteria have been reported,35 and the oxidation and oxidative processes of low-density lipoprotein (LDL) are considered important to the pathogenesis of arteriosclerosis.45 Therefore, by preventing the oxidation of LDL, it may be possible to reduce the incidence of atherosclerosis. It has not been confirmed whether lactic acid bacteria actually prevent the oxidation of LDL, although Zommana *et al.* have reported these bacteria to have antioxidative properties.7,9 Such properties would suggest that the oxidation of LDL could be inhibited by the consumption of a culture of lactic acid bacteria.

We confirm in this study that radical scavengers were produced in the culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2038. Moreover, we show that the ethereal extract from this culture inhibited the oxidation of the membrane prepared from rabbit erythrocytes *in vitro*. We finally show that the administration of freeze-dried powder of the 2038 culture prevented the oxidation of lipoprotein in rats.

**Materials and Methods**

*Strain and culture*. The bacterial strain used in this study was *Lactobacillus delbrueckii* subsp. *bulgaricus* 2038, a starter of yoghurt. A culture of strain 2038 was obtained by cultivating with a skim milk medium (10% skim milk powder in distilled water) at 37°C for 24 h.

*Extract from the culture*. The supernatant (5 ml) of the culture of strain 2038 was extracted twice with ether (5 ml). The resulting ethereal extract was evaporated to dryness, dissolved in methanol (0.2 ml), and used as a sample for the detection of radical scavengers and for an antioxidative assay.

*Detection of radical scavengers*. The extract (2 μl) was subjected to TLC (plate, silica gel 60, Merck; developing solvent, chloroform:methanol = 8:2). The patterns of spots on the plate were visualized by soaking the plate in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (800 μg/ml in ethanol) for a few seconds. DPPH is a radical that has a purple color, this color being lost as the ability of DPPH to act as a radical scavenger diminishes; therefore, radical scavengers on the plate appeared as spots due to the decoloration of DPPH.13

*Antioxidative assay*. This assay was performed

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Abbreviations: LDL, low-density lipoprotein; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances
according to the method reported by Osawa.\textsuperscript{12-13} The membrane (0.85 ml) obtained from rabbit erythrocytes was added to 0.1 ml of the extract from the supernatant of the 2038 culture (24 h), and t-butylhydro-peroxide (0.05 ml) was added to the reaction mixture to start the oxidation. The reaction mixture was incubated at 37°C for 15 min, after which the level of thiobarbituric acid-reactive substances (TBARS) in the reaction mixture was measured by the TBA method.\textsuperscript{14} As a control sample, the extract from the supernatant of the skim milk medium containing skim milk (10%) and lactic acid (1%) was used. Vitamin E solutions (0–1000 μg/ml) were used as positive control samples.

\textit{In vivo study.} The care of the rats in this study was done according to the Standards Relating to the Rearing and Keeping of Industrial Animals (the Prime Minister’s Office, Japan).

Male F344 rats at 11 weeks old (Japan SLC) were divided into 5 groups of 7 animals each: fresh oil—skim milk group, fresh oil—L.2038 (freeze-dried powder of the culture of strain 2038) group, oxidized oil—skim milk group, oxidized oil—L.2038 group, and control group. Each group of animals was fed on their respective diet for 4 weeks. The compositions of the diets are shown in Table 1. The diet for the control group was based on the AIN93G formula with the protein source modified; skim milk powder (34% protein content) and casein were used as the sources of protein.

Oxidized oil was prepared by heating fresh soybean oil at 60°C for 9 days (peroxide value, 91 meq; TBARS, 118 nmol/ml).

Blood was withdrawn from the abdominal aorta under light diethyl ether anesthesia and collected in heparin-coated tubes. Plasma was then prepared from the blood by centrifugation (1000 g, 10 min), and the level of vitamin E in the plasma was determined. This plasma was used for the preparation of LDL.

\textit{Determination of vitamin E.} The level of vitamin E in the plasma was determined according to the method reported by Jaffar.\textsuperscript{15}

\textit{Lipoprotein analysis.} Lipoprotein was prepared by ultra-centrifugation as described by Naito,\textsuperscript{16} and its cholesterol content was determined with a kit (Cholesterol C test Wako, Wako Pure Chemical Industries). The level of TBARS in the lipoprotein was determined according to the method reported by Naito.\textsuperscript{17} The TBA solution (0.67%, 1 ml) and HCl (0.05 N, 3 ml) were added to the lipoprotein fraction (0.3 ml), and this reaction mixture was heated for 30 min in a boiling water bath. After the mixture had been cooled in an ice bath, TBARS in the reaction mixture were extracted with 4 ml of n-butanol containing 15% methanol. The absorbance of the butanol phase was measured at 535 nm. A standard curve was prepared for malondialdehyde, and the level of TBARS is expressed as malondialdehyde equivalents/mg of cholesterol.

\textit{Statistical analysis.} Each result is expressed as the mean ± SD. One-way ANOVA was used to examine whether there were differences among the four groups, and when a statistically significant effect was observed (p<0.05), Fisher’s PLSD was used to compare among the groups.

\section*{Results}

\textit{Radical scavengers in the culture of strain 2038}\n
Spots appeared on the plate (Fig. I), those not detected in the extract from the skim milk medium (0 h) being detected in the extract from the culture (24 h). The extent of decoloration of the spots depended on the cultivation time. These spots showed that radical scavengers were produced in the culture of strain 2038.

\textit{Extract from the culture and its anti-oxidative effect}\n
The yields of the extract for the anti-oxidation assay from the supernatant (5 ml) of the culture and from the skim milk medium were 3.8 mg and 0.4 mg, respectively. These values show that 3.4 mg of the extract from the supernatant of the culture had been produced by strain 2038. The vitamin E contents were less than 1 μg/ml (0.17 μg/ml in the medium and 0.15 μg/ml in the culture).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Component} & \textbf{Control group} & \textbf{Fresh oil-skim milk group} & \textbf{Fresh oil-L.2038 group} & \textbf{Oxidized oil-skim milk group} & \textbf{Oxidized oil-L.2038 group} \\
\hline
Fermented product & & & & & \\
Skim milk\textsuperscript{a} & 100.0 & 100.0 & 100.0 & 100.0 & 100.0 \textsuperscript{a} \\
Corn starch\textsuperscript{b} & 463.5 & 333.5 & 333.5 & 333.5 & 333.5 \textsuperscript{b} \\
Casein\textsuperscript{c} & 166.0 & 166.0 & 166.0 & 166.0 & 166.0 \textsuperscript{c} \\
Sucrose\textsuperscript{d} & 100.0 & 100.0 & 100.0 & 100.0 & 100.0 \textsuperscript{d} \\
Fresh soybean oil\textsuperscript{e} & 70.0 & 200.0 & 200.0 & & \\
Oxidized soybean oil & & & & 200.0 & 200.0 \\
Fiber\textsuperscript{f} & 50.0 & 50.0 & 50.0 & 50.0 & 50.0 \textsuperscript{f} \\
Mineral mix\textsuperscript{g} & 35.0 & 35.0 & 35.0 & 35.0 & 35.0 \textsuperscript{g} \\
Vitamin mix\textsuperscript{h} & 10.0 & 10.0 & 10.0 & 10.0 & 10.0 \textsuperscript{h} \\
Choline bitartrate\textsuperscript{i} & 2.5 & 2.5 & 2.5 & 2.5 & 2.5 \textsuperscript{i} \\
t-Cystine\textsuperscript{j} & 3.0 & 3.0 & 3.0 & 3.0 & 3.0 \textsuperscript{j} \\
\hline
\end{tabular}
\caption{Composition of the Diets (g/kg of diet)}
\end{table}

\textsuperscript{a}Meiji Milk Products Co., Tokyo; \textsuperscript{b}Nihon Shokuhin Kakou Co., Tokyo; \textsuperscript{c}New Zealand Milk Product Co., Wellington; \textsuperscript{d}Dai-Nippon-Mejii Sugar Co., Tokyo; \textsuperscript{e}Wako Pure Chemical Industries Co., Osaka; \textsuperscript{f}Asahi Chemical Industry Co., Osaka; \textsuperscript{g}AIN93G formula.
Fig. 1. Radical Scavengers Produced by Strain 2038.

Ethereal extracts from the 2038 culture cultivated for various periods (0–24 h) were subjected to TLC. The background color (purple) of the chromatogram was due to DPPH (1,1-difenil-2-picrylhidrazilo).

The level of TBARS in the erythrocyte membrane incubated with the extract from the supernatant of the culture was determined. The level was lower in the erythrocytes incubated with the supernatant of the culture than with the supernatant of the skim milk medium (Table 2). This result shows that the anti-oxidative activity of the culture supernatant of strain 2038 was stronger than that of the supernatant of the skim milk medium.

In vivo study

There was no difference in body weight among the rats of the five groups, indicating that the type of diet did not affect the body weight gain. There was no difference in the cholesterol level of the VLDL and LDL fractions between the oxidized oil—skim milk group and oxidized oil—L2038 group (Fig. 2). However, the TBARS value for the VLDL fraction of the oxidized oil—skim milk group was higher than that of any other group (Fig. 3). Similarly, the TBARS value for the LDL fraction of the oxidized oil—skim milk group was higher than that of any other group (Fig. 4). However, in respect of the Lp (α) and HDL fractions, there was no difference in the level of TBARS between the oxidized oil—skim milk group and oxidized oil—L2038 group (Figs. 5 and 6).

The serum vitamin E level was lower in the oxidized oil—skim milk group and oxidized oil—L2038 group than in the fresh oil—skim milk group and fresh oil—L2038 group. However, the level was higher in the oxidized oil—L2038 group than in the oxidized oil—skim milk group (Fig. 7).

Fig. 2. Cholesterol Levels in Lipoprotein.

Blood was taken from a rat of each group: C, control group; FS, fresh oil—skim milk group; FL, fresh oil—L2038 (freeze-dried powder of the strain 2038 culture) group; OS, oxidized oil—skim milk group; OL, oxidized oil—L2038 group. The lipoprotein fractions were separated by ultra-centrifugation, and the cholesterol level in each fraction was measured (mean ± SD). Bars with different letters are significantly different (p<0.05) based on Fisher’s PLSD.

Fig. 3. Level of TBARS in VLDL.

Blood was taken from a rat of each group, and the very low-density lipoprotein (VLDL) fraction was collected from the serum. Bars (mean±SD) with different letters are significantly different (p<0.05) based on Fisher’s PLSD.

Table 2. Antioxidative Effect of Strain 2038 on the Oxidation of the Erythrocyte Membrane

<table>
<thead>
<tr>
<th>Sample</th>
<th>tBHP</th>
<th>TBARS (MDA: mmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>+</td>
<td>7.07</td>
</tr>
<tr>
<td>Distilled water</td>
<td>−</td>
<td>0.29</td>
</tr>
<tr>
<td>Vitamin E in methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 µg/ml</td>
<td>+</td>
<td>7.28</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>+</td>
<td>6.45</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>+</td>
<td>5.52</td>
</tr>
<tr>
<td>1000 µg/ml</td>
<td>+</td>
<td>0.56</td>
</tr>
<tr>
<td>Extract in methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skim milk medium</td>
<td>+</td>
<td>3.04</td>
</tr>
<tr>
<td>Culture of strain 2038 (24 h)</td>
<td>+</td>
<td>1.36</td>
</tr>
</tbody>
</table>

Ethereal extracts were used as samples and incubated with the erythrocyte membrane and a t-butyl-hydroperoxide (tBHP) solution at 37°C for 15 min. The TBARS value of the membrane was measured after incubation.
Fig. 4. Level of TBARS in LDL.
Blood was taken from a rat of each group, and the low-density lipoprotein (LDL) fraction was collected from the serum. Bars (mean ± SD) with different letters are significantly different (p<0.05) based on Fisher’s PLSD.

Fig. 5. Level of TBARS in Lp(a).
Blood was taken from a rat of each group, and the lipoprotein (a) (Lp(a)) fraction was collected from the serum. Bars (mean ± SD) with different letters are significantly different (p<0.05) based on Fisher’s PLSD.

Fig. 6. Level of TBARS in HDL.
Blood was taken from a rat of each group, and the HDL high-density lipoprotein (HDL) fraction was collected from the serum. Bars (mean ± SD) with different letters are significantly different (p<0.05) based on Fisher’s PLSD.

Discussion
While many reports have documented the anti-oxidative effect of vegetables, only a few studies have been made on the anti-oxidative effect of lactic acid bacteria. One study has reported that some species of Lactobacillus had an anti-oxidative effect, although Lactobacillus delbrueckii subsp. bulgaricus, which is used as a starter for yoghurt, was not among them. In the present study, the anti-oxidative effect of the culture of Lactobacillus delbrueckii subsp. bulgaricus 2038 was investigated. Radical scavengers were detected in the culture by DPPH, and the culture was also shown to prevent oxidation of the erythrocyte membrane. The anti-oxidative ability of the extract from the culture of strain 2038 (24 h) was stronger than that of 100 μg/ml of vitamin E (Table 2). Although the extract from the skim milk medium had also anti-oxidative ability, the anti-oxidative ability of the extract from the culture was much stronger. These findings suggest that Lactobacillus delbrueckii subsp. bulgaricus had an anti-oxidative effect.

The consumption of vegetables, tea, and wine has been reported to prevent atherosclerosis. The oxidation of LDL plays an important role in the pathogenesis of atherosclerosis, and the antioxidants in these foods inhibit this oxidation. Similarly, the consumption of the culture of strain 2038 may also prevent the oxidation of LDL. In the present study, the culture of strain 2038 was also found to have a preventative effect on LDL oxidation in vivo. The level of TBARS in the LDL fraction obtained from rats fed on the oxidized soybean oil was higher than that in the rats fed on fresh soybean oil, as one study had shown. However, the TBARS value for the LDL fraction of the rats fed on both oxidized oil and the freeze-dried culture of strain 2038 was lower than the value for the rats fed on skim milk, even though there was no difference in the serum cholesterol level of the LDL fraction among the groups, except for the control group. The oxidation of LDL was thus prevented in vivo by administration of the freeze-dried culture of strain 2038. Furthermore, the level of vitamin E in the serum was also affected by the diet: it was lower in the rats fed on oxidized oil than in the rats fed on fresh oil, and higher in the rats fed on the
freeze-dried culture of strain 2038 than in the rats fed on skim milk. The vitamin E contents in the medium and the culture were both very low. On the other hand, the antioxidative ability of the culture was stronger than that of the medium, the difference being equivalent to about 100 μg/ml of vitamin E (This value was estimated from the vitamin E data in Table 2). This result shows that the anti-oxidative ability in the diet containing freeze-dried powder of the culture of strain 2038 was stronger than that in the diet containing the skim milk powder (This difference was estimated to be equivalent to about 4 mg of vitamin E in 1 kg of the AIN93G mixture, because 0.2 ml of the extract was prepared from 5.0 ml of a culture that contained 10% (v/w) of solid ingredients in vitro, and the freeze-dried powder content of the culture of strain 2038 in the diet was 10% (w/w) in vivo). It has been reported that the plasma vitamin E level in rats was elevated by 45 mg/kg of vitamin E in the diet. It has also been reported that vitamin E supplementation (100 mg/day) to elderly people prevented the oxidation of linoleic acid in LDL. Therefore, while the antioxidant in the culture of strain 2038 had an antioxidative effect similar to that of vitamin E, it is very difficult to compare the antioxidative ability of lactic acid bacteria with that of other antioxidants because of their different characteristics, absorption in the intestines, and metabolism. Some of the antioxidants have been purified and examined; for example, catechin in tea was incorporated into human plasma, and contributed to a high plasma antioxidative capacity. In respect of the antioxidant in the culture of lactic acid bacteria, its chemical structure, characteristics, absorption in the intestines, and metabolism in the human body are currently unknown. Further examination of the antioxidants produced by lactic acid bacteria will elucidate this.

In summary, the culture of Lactobacillus delbrueckii subsp. bulgaricus 2038, a starter strain for yoghurt, exhibited antioxidative activity and prevented the oxidation of LDL in vivo.

References

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