Metabolic Fate and Expectation of Health Benefits of [U-14C]-Sucrose Inhibited from Digestion Using Morus alba Leaf Extract

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Summary Morus alba leaf extract (MLE), a strong inhibitor of sucrase, suppresses blood glucose elevation following sucrose ingestion. To investigate that sucrose inhibited from digestion using MLE is utilized through gut microbiota, [U-14C]-sucrose solutions with or without MLE were administered orally to conventional and antibiotic-treated rats, and the excretion of 14CO2 and H2 produced by gut microbiota was measured for 24 h. After an administration of [U-14C]-sucrose to conventional rats, the unit excreted 14CO2 peaked at 4 h, and the cumulative 14CO2 excreted over 24 h was approximately 60% of the radioactivity administered. No H2 was excreted. Following an administration of [U-14C]-sucrose and MLE in conventional rats, the unit excreted 14CO2 peaked later, at 8 h, and was significantly lower (p<0.05). The cumulative 14CO2 excreted over 24 h was equal in both groups, although there was a time lag of 2–3 h in rats given [U-14C]-sucrose and MLE. The amount of H2 excreted by these rats peaked 8 h after administration. Following the administration of [U-14C]-sucrose and MLE to antibiotic-treated rats, the unit excreted 14CO2 peaked lower, and the cumulative 14CO2 excretion over 24 h was approximately 40%. In this group, H2 was minimally excreted. H2 and 14CO2 produced by gut microbiota were excreted simultaneously. In conclusion, sucrose inhibited from digestion using MLE was fermented spontaneously by gut microbiota and was not excreted into feces. In addition, it confirmed that H2 excretion could be used directly to indicate the degree of fermentation of nondigestible carbohydrates.

Key Words 14C-sucrose, Morus alba leaf extract, gut microbiota, hydrogen excretion, metabolic fate

Morus alba leaves include 1-deoxynojirimycin (DNJ) and its derivatives, which strongly inhibit α-glucosidases such as sucrase and maltase (1–4). Therefore, sucrose ingested simultaneously with Morus alba leaf extract (MLE) does not elevate postprandial blood glucose and insulin levels in humans and rats, while it increases the excretion of hydrogen (H2), a specific product of gut microbiota (5–11). The excretion of H2 suggests that sucrose inhibited from digestion by MLE is fermented by gut microbiota and expected to improve gut microflora.

Nondigestible carbohydrates, such as dietary fiber, fructooligosaccharide (FOS) and galactooligosaccharide (GOS), do not raise postprandial blood glucose and insulin levels following ingestion, but they are partially or completely fermented by gut microbiota and produced short-chain fatty acids (SCFAs), carbon dioxide (CO2), H2, methane (CH4) and others (12–15). Under the active fermentation as following FOS ingestion, a lot of SCFAs are produced in the large intestine, and a large amount of extracorporeal excretion of H2 is detected in humans and rats (15–19). SCFAs which are produced by gut microbiota, change the composition of gut microbiota and bring about the improvement of environment of gastrointestinal tract (20). Furthermore, SCFAs produced by gut microbiota are spontaneously absorbed in the large intestine and further metabolized into CO2 to produce energy for the host (21).

We have proposed the indirect and simple evaluation method for determining the relative available energy of

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Abbreviations: AUC, area under the curve; DNJ, 1-deoxynojirimycin; FOS, fructooligosaccharide; GOS, galactooligosaccharide; MLE, Morus alba leaf extract; SCFAs, short-chain fatty acids.
nondigestible carbohydrates based on fermentability using \( H_2 \) excretion and tried to evaluate those of some dietary fiber materials (16–19, 22, 23). To evaluate the relative available energy of nondigestible carbohydrates, the fermentability is essential for estimating the amount of SCFAs produced by gut microbiota. We have been using relative fermentability estimated from \( H_2 \) excretion in the breath. The \( CO_2 \) excreted from the body contains not only \( CO_2 \) produced by fermentation, but also \( CO_2 \) produced by oxidation of nutrients absorbed in the small intestine. Therefore, \( CO_2 \) excretion cannot be used to estimate the fermentability. In the present study, we are going to confirm that the dynamics of \( H_2 \) and \( CO_2 \) produced by gut microbiota are simultaneously excreted from the body, and \( H_2 \) excretion can be used as an indicator of fermentability to evaluate the relative available energy of nondigestible carbohydrates. Hydrogen excretion is also increased, when sucrose is ingested simultaneously with \( \alpha \)-glucosidase inhibitors such as acarbose and miglitol (24–30).

The objective of the present study is to clarify whether the sucrose prevented from digestion with MLE is metabolized spontaneously by gut microbiota as FOS and utilized as an energy source. In addition, it is investigated whether \( H_2 \) and \( CO_2 \) excretion would show similar trends if the sucrose is utilized by gut microbiota. Then, after \([U-14C]\)-sucrose solutions with or without MLE were orally administrated to conventional and antibiotic-treated rats, we measured the excretion of \( ^14CO_2 \) and \( H_2 \) produced by gut microbiota for 24 h, respectively. Furthermore, the incorporation of \(^{14}C\)-radioactivity into tissues was investigated.

**MATERIALS AND METHODS**

*Ethics approval and informed consent.* The protocol for animal studies was approved (receipt no. 23-12, approval no. 23-11) by the Committee on Animal Experiments of the University of Nagasaki (Nagasaki, Japan). These experiments were conducted according to the Guidelines on the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and the standards related to the Care and Management of Experimental Animals (notification number 88, from the Prime Minister’s Office, Tokyo, Japan). All experiments were carried out in the Public Health Nutrition Laboratory and the radiation unit control area of the Graduate School of Human Health Science, University of Nagasaki Siebold.

*Radioactivity.* \([U-14C]\)-sucrose (specific activity of 22.2 GBq/mmol, 600 mCi/mmol, 1 mCi/mL in 90% ethanol, >99% purity; American Radiolabeled Chemicals, Inc., MO, USA) at 250 \( \mu \)Ci/250 \( \mu \)L was used in the experiments.

*Morus alba leaf extract.* MLE was provided by Toyotama Healthy Food Co. Ltd. (Tokyo, Japan). It was prepared as follows: dried *M. alba* leaves were extracted with nine volumes of 50% (w/v) ethanol for 2 h at 23°C under the mixing hood, and ethanol was removed with an evaporator. The crude extract from the *Morus alba* leaves was purified with column chromatography using a cation-exchange resin to remove impurities and the obtained extract solution was concentrated. The original MLE solution contained 0.24% DNJ equivalents, including derivatives (Table 1). MLE has a green grass flavor and is khaki or dark green in color. It was determined to be safe (31).

*Preparation of test solutions containing \([U-14C]\)-sucrose.* The test solutions containing \([U-14C]\)-sucrose were prepared as follows: 159 kBq (4.3 \( \mu \)Ci) of \([U-14C]\)-sucrose and 300 mg of sucrose were dissolved in 2 mL of sterilized water, on the base of our previous experiments (21, 32–34). The solutions containing both \([U-14C]\)-sucrose and MLE were prepared as follows: 159 kBq (4.3 \( \mu \)Ci) of \([U-14C]\)-sucrose and 300 mg of sucrose were dissolved in 2 mL of original MLE solution containing 0.24% DNJ equivalents. The radioactivity incorporated into feces, urine, tissue, and organs was measured using a scintillation counter. The MLE solution amount (2 mL) was enough for significant inhibition of sucrose in the digestion of sucrose (300 mg) (2, 3). For the administration of \([U-14C]\)-sucrose in antibiotic-treated rats, the test solution was passed through a membrane filter (\( 0.22 \mu \text{m}, \) Millipore Corp., MA, USA) to remove any bacteria. A test solution containing 300 mg of sucrose without \([U-14C]\)-sucrose was used in the experiment to confirm the suppressive effect of MLE on blood glucose elevation.

*Animals, housing, and diet.* To confirm the suppressive effect of MLE on blood glucose elevation, five male Wistar rats (average body weight of 250 g; CLEA Japan, Inc., Tokyo, Japan) were given a control diet and sterilized water ad libitum for 3 d, and then were orally given sucrose solution (300 mg/2 mL) after fasting for 15 h. Blood samples (100 \( \mu \)L) were collected from a tail vein using a hematocrit tube 0, 30, 60, 90, 120, 150, and 180 min after oral administration of the test solutions, and serum was obtained by centrifugation at 2,500 \( \times g \) for 20 min at 20°C (High Speed Refrigerated Centrifuge 6900, Kubota Co., Tokyo, Japan). Glucose concentration in serum was measured with the Trinder’s method (35). The control diet was an AIN93M diet (36), with the cellulose replaced with \( \beta \)-cornstarch to exclude its effect on gut microbiota.

To deplete the gut microbiota, seven male Wistar rats (average body weight: 250 g, CLEA Japan, Inc.) were given the control diet and the sterilized water containing four kinds of antibiotics ad libitum for 10 d before

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**Table 1. Analytical table of *Morus alba* leaf extract.**

<table>
<thead>
<tr>
<th>Analyzed category</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid content</td>
<td>0.6%</td>
</tr>
<tr>
<td>1-Deoxynojirimycin content</td>
<td>0.24%</td>
</tr>
<tr>
<td>Arsenic</td>
<td>&lt;2.0 ppm</td>
</tr>
<tr>
<td>Heavy metal</td>
<td>&lt;20.0 ppm</td>
</tr>
<tr>
<td>Live bacteria count</td>
<td>90/g</td>
</tr>
<tr>
<td><em>E. coli</em> count</td>
<td>negative</td>
</tr>
<tr>
<td>Mold · Yeast</td>
<td>30/g</td>
</tr>
</tbody>
</table>
the experiments (21, 32). The sterilized water (1 mL) contained 0.5 mg of vancomycin hydrochloride (Shionogi Pharmaceutical Co., Ltd., Tokyo, Japan), 1.0 mg of sodium ampicillin, 1.0 mg of neomycin sulfate, and 1.0 mg of metronidazole (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The other 10 Wistar male rats (average body weight of 270 g; CLEA Japan, Inc.) were given the control diet and distilled water ad libitum for 7 d and were administered [U-14C]-sucrose solutions with or without MLE. Rats were housed in individual stainless-steel cages and kept at room temperature (22–24°C). The relative humidity was maintained at 50–55% with a 12 h light/dark cycle (light, 08:00–20:00; dark, 20:00–08:00). The rats were starved for 15 h before administration of the test solution.

Confirmation of gut microbiota depletion. To confirm the depletion of gut microbiota, two out of seven rats that were given drinking water containing the four kinds of antibiotics for 7 d were killed 3 d before [U-14C]-sucrose administration, and cecal contents were removed anaerobically. The cecal contents were weighed and centrifuged at 1,500 ×g for 15 min with PBS buffer solution in twice. The evaluation of gut microbiota depletion from the cecal content was carried out according to Mitsuoka’s method (37) using BL medium and thioglycolate broths I and II (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), which are non-selective media. The media were inoculated with the cecal contents and were incubated for 24, 48, and 72 h at 37°C under aerobic and anaerobic conditions to observe bacterial growth.

Oral administration of [U-14C]-sucrose to measure the incorporation of 14C radioactivity in carbon dioxide, gastrointestinal contents, feces, urine, tissues, and organs in conventional and antibiotic-treated rats. To measure the radioactivity converted to 14CO2 or incorporated into tissues from orally administered [U-14C]-sucrose, the rats were starved and only supplied drinking water for 15 h before the administration of the test solutions. Five conventional rats were given the test solution (2 mL) containing 159 kBq of [U-14C]-sucrose and 300 mg of sucrose, and other five conventional rats were given the test solution (2 mL) containing 159 kBq of [U-14C]-sucrose, 300 mg of sucrose, and MLE (containing 4.8 mg of DNJ equivalents), using a stomach tube. To investigate the metabolism of [U-14C]-sucrose via gut microbiota following sucrose inhibition from digestion by MLE, five antibiotic-treated rats were also administered the test solution (2 mL) containing 159 kBq of [U-14C]-sucrose, 300 mg of sucrose, and MLE (containing 4.8 mg of DNJ equivalents). The radioactivity of test solution was determined with a scintillation counter. Before administration, rats which were administered with test solution, were transferred into a glass metabolic apparatus with five containers (Metabolica CO-2; Sugiyamagen Co. Ltd., Tokyo, Japan). The interior temperature was maintained at 23±1°C. The excreted 14CO2, urine, and feces were collected for 24 h after administration of the test solution, as in our previous experiments (21, 32–34). The rats that were transferred to the glass metabolic apparatus were made to fast for further 6 h and only given drinking water. 14CO2 excreted from the rats was trapped with 500 mL of mono-ethanolamine (FUJIFILM Wako Pure Chemical Corporation), and 2 mL of mono-ethanolamine was removed to measure the radioactivity at 2, 4, 6, 8, 10, 12, and 24 h after administration. The excreted 14CO2 was trapped (absorbed) with mono-ethanolamine for 12 h after administration of the test solution, and the glass container was replaced with a new one with 500 mL of fresh mono-ethanolamine. 14CO2 collection was continued for an additional 12 h.

Feces and urine were separately collected in each glass bottle fixed to the metabolic apparatus, and these bottles were changed to new bottles at 2, 4, 6, 8, 10, and 12 h after administration for 14CO2 determination. The rats were killed by decapitation at 24 h after administration of the test solution, and the blood was collected in a glass tube. After the abdomen was opened, the contents and tissues of the stomach and small intestine, cecum and colon, liver, kidneys, gastrocnemius muscle of the right leg, perirenal and epididymal adipose tissues, as well as small intestinal mucosa, were all removed to measure the incorporated radioactivity.

Sample preparation for measuring 14C-radioactivity was carried out as described in our previous experiments (21, 32–34). Five hundred microliters of mono-ethanolamine with trapped 14CO2 were transferred into a counting vial, and the radioactivity was determined with a liquid scintillation counter (LSC-6000; Aloca Co. Ltd., Tokyo, Japan) after the addition of 5 mL of scintillation cocktail (Hionic-Fluor; Perkin Elmer Japan Co. Ltd., Kanagawa, Japan) and 1 mL of cellosolve (FUJIFILM Wako Pure Chemical Corporation). All obtained the tissue, organ, stomach content, small intestine, cecum, colon, and feces were homogenized in nine volumes (10%) of cold 0.9% NaCl solution using a polytron homogenizer (Kinematica Co. Ltd., Lucerne, Switzerland). Three hundred microliters of homogenates or serum were transferred into a counting vial and solubilized with 0.5 mL of solubilizer (Soluene 350; Perkin-Elmer Japan Co. Ltd., Tokyo, Japan) at 50°C for 12 h. The feces and contents of the cecum and colon were decolorized by adding 0.3 mL of 30% hydrogen peroxide (FUJIFILM Wako Pure Chemical Corporation). Five milliliters of scintillation cocktail (Pico-fluor 40, Perkin-Elmer Japan Co. Ltd.) were added to the solubilized homogenates and urine in the counting vial, and the radioactivity was determined with a scintillation counter. The counting efficiency was approximately 90%.

Calculation and statistical analysis. The radioactivity recovered in 14CO2, tissue, organs, and the contents and tissue of the gastrointestinal tract was calculated as a percentage to the radioactivity administered orally. The radioactivity of the 14CO2 that was trapped cumulatively with mono-ethanolamine during the first 12 h and between 12 h and 24 h after administration of the test solutions were added together. Data were calculated as means and standard errors. After the normal distribution test, the data were analyzed by repeated mea-
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\[ \text{Fate of } 14\text{C-Sucrose Inhibited from Digestion by } \text{Morus alba} \]

sures two-way ANOVA. Student \( t \)-test was used to compare between conventional rats given \([U-14\text{C}]-\text{sucrose with and without MLE for the time-dependent data. The percentage of the resulting radioactivity after 24 h versus dosed radioactivity was compared between conventional rats given } [U-14\text{C}]-\text{sucrose with and without MLE by Student } t\text{-test. The } 14\text{CO}_2 \text{ produced by fermentation of gut microbiota of sucrose that prevented from digestion with MLE calculated as a percentage of each value from conventional and antibiotic-treated rats given } [U-14\text{C}]-\text{sucrose solutions with MLE. A } p \text{ value less than } 0.05 \text{ was considered significant. The statistical analysis was performed using SPSS version 21 for Windows (SPSS Inc., Tokyo, Japan) (38). Sample size of each experiment was conducted based on our previous studies (3, 21, 39).}

\[ \text{RESULTS} \]

\textit{Suppression of blood glucose elevation by Morus alba leaf extract after sucrose administration in conventional rats}

When the test solution (2 mL) containing 300 mg of sucrose and MLE (containing 4.8 mg of DNJ equivalents) was administered to conventional rats, serum glucose elevation was suppressed significantly at 30 and 60 min \((p<0.05)\) before returning back to basal levels at 120 min after administration (Fig. 1). But, the blood glucose elevation was not inhibited completely by DNJ.

\[ \text{14CO}_2 \text{ excretion after administration of } [U-14\text{C}]-\text{sucrose solutions with and without Morus alba leaf extract in conventional and antibiotic-treated rats} \]

\([U-14\text{C}]-\text{sucrose solutions (159 kBq of } [U-14\text{C}]-\text{sucrose and 300 mg of sucrose in 2 mL} \text{) with or without MLE (containing 4.8 mg of DNJ equivalents) were administered orally to conventional and antibiotic-treated rats after fasting for 15 h, and the rate of } 14\text{CO}_2 \text{ excretion was measured at 2-h intervals for 12 h and then again at 24 h (Fig. 2-A). When the } [U-14\text{C}]-\text{sucrose solution without MLE was administered orally to conventional rats, } 14\text{CO}_2 \text{ excretion started to increase in an early stage and showed a peak at 4 h, and it then decreased until 12 h. After the } [U-14\text{C}]-\text{sucrose solution with MLE was administered to conventional rats, the rate of } 14\text{CO}_2 \]

\[ \text{Fig. 1. Suppression of } \text{Morus alba extract for blood glucose elevation by sucrose administration in conventional rats. Sucrose solutions (300 mg in 2 mL) with or without MLE (DNJ equivalent 4.8 mg) was orally administered to conventional rats after fasting for 15 h, and blood (100 } \mu\text{L}) \text{ was collected from the tail vein. ○, administration of sucrose alone (} n = 5); ●, administration of sucrose and MLE (} n = 5). MLE, } \text{Morus alba extract. Data were expressed as the mean}\pm\text{S.E. * Significantly lower versus sucrose alone at each time point, at } p<0.05 \text{ by repeated measures two-way ANOVA and, Student } t\text{-test, respectively.}

\[ \text{Fig. 2. Profile } 14\text{CO}_2 \text{ excretion after oral administration of } [U-14\text{C}]-\text{sucrose solutions with and without } \text{Morus alba leaf extract to conventional and antibiotic-treated rats. [U-14\text{C}]-sucrose solutions (159 kBq: 300 mg in 2 mL) with or without MLE (DNJ equivalent 4.8 mg) were orally administered to conventional and antibiotic-treated rats after fasting for 15 h. The rats were moved immediately to a metabolic apparatus to trap } 14\text{CO}_2 \text{ and absorbent (0.5 mL) trapped } [14\text{C}]-\text{radioactivity was collected at 2 h intervals until 12 h and at 24 h after administration. ○, Administration of sucrose alone to conventional rats (} n = 5); ●, administration of sucrose and MLE to conventional rats (} n = 5); ●, administration of sucrose and MLE to antibiotic-treated rats (} n = 5). MLE, } \text{Morus alba leaf extract. Data are expressed as the mean}\pm\text{S.E. * Significant differences between conventional rats given } [U-14\text{C}]-\text{sucrose with and without MLE were evaluated at each time point at } p<0.05 \text{ by repeated measures two-way ANOVA and, Student } t\text{-test, respectively.} \]
excretion was delayed and had a peak at 8 h. The rates of $^{14}$CO$_2$ excretion were significantly lower at 2 h and 4 h, and significantly higher at 8, 10, and 12 h as compared with the rates in rats receiving the [U-$^{14}$C]-sucrose solution without MLE ($p<0.05$) (Fig. 2-A). However, the areas under the curve (AUC) of the $^{14}$CO$_2$ excretion curves over 12 h were similar between the two groups. In the first 4 h after [U-$^{14}$C]-sucrose and MLE were administered simultaneously ($p<0.05$). In addition, there were significant differences between conventional rats given [U-$^{14}$C]-sucrose with and without MLE were evaluated at each time point at $p<0.05$ by repeated measures two-way ANOVA and Student $t$-test, respectively.

Figure 2-B shows the cumulative $^{14}$CO$_2$ excretion at 1-h intervals until 12 h and then again 24 h after administration of [U-$^{14}$C]-sucrose solutions with and without Morus alba leaf extract to conventional and antibiotic-treated rats. After [U-$^{14}$C]-sucrose solution was orally administered to conventional and antibiotic-treated rats, $^{14}$CO$_2$ was excreted very slowly until 24 h. The cumulative $^{14}$CO$_2$ excretion was 40% of the total radioactivity administered in these rats.

Hydrogen excretion after administration of [U-$^{14}$C]-sucrose solutions with and without Morus alba leaf extract in conventional and antibiotic-treated rats

Figure 3 shows $H_2$ excretion at 1-h intervals until 12 h and then at 24 h after conventional and antibiotic-treated rats were given [U-$^{14}$C]-sucrose solutions with or without MLE. When [U-$^{14}$C]-sucrose alone was administered orally to conventional rats, $H_2$ excretion was minimally observed. However, in conventional rats administered [U-$^{14}$C]-sucrose solution with MLE, $H_2$ was excreted slowly in the early period and then excreted more rapidly until 8 h after administration. Excretion then decreased rapidly until 12 h and was not detectable at 24 h. In contrast, when the [U-$^{14}$C]-sucrose solution with MLE was administered orally to antibiotic-treated rats, $H_2$ excretion was minimally observed for 24 h.

$^{14}$C radioactivity in gastrointestinal contents and feces 24 h after administration of [U-$^{14}$C]-sucrose solutions with and without Morus alba leaf extract in conventional and antibiotic-treated rats

Conventional and antibiotic-treated rats that were orally administered [U-$^{14}$C]-sucrose solutions with or without MLE were killed 24 h after administration, and the incorporated $^{14}$C-radioactivity was determined (Table 2).

Figure 4 shows the $^{14}$C-radioactivity recovered from the contents of the gastrointestinal tract and feces 24 h after the test solution is administered in conventional and antibiotic-treated rats. They were calculated in three parts: stomach and small intestine (upper intestine); cecum, colon, and rectum (lower intestine); and feces. The amount of $^{14}$C-radioactivity recovered in the lower intestine of conventional rats was less when given [U-$^{14}$C]-sucrose alone compared with [U-$^{14}$C]-sucrose and MLE simultaneously ($p<0.05$). In addition, there was significantly less $^{14}$C-radioactivity in the feces of conventional rats administered [U-$^{14}$C]-sucrose alone as compared with that of conventional rats administered [U-$^{14}$C]-sucrose solution with MLE ($p<0.05$). $^{14}$C-radioactivity remained high in the cecum, colon, and rectum of antibiotic-treated rats, the amount of $^{14}$C-radioactivity in feces was not.

Tissue distribution of $^{14}$C radioactivity 24 h after administration of [U-$^{14}$C]-sucrose solutions with and without Morus alba leaf extract in conventional and antibiotic-treated rats

The radioactivity incorporated into tissues and organs was not significantly different among conventional rats given [U-$^{14}$C]-sucrose, conventional rats given [U-$^{14}$C]-sucrose and MLE (Table 2). The total

![Fig. 3. Profile of $H_2$ excretion after administration of [U-$^{14}$C]-sucrose solutions with or without Morus alba leaf extract to conventional and antibiotic-treated rats. Rats administered [U-$^{14}$C]-sucrose solutions (159 kBq, 300 mg in 2 mL) with and without MLE (4.8 mg DNJ equivalent) are the same in Fig. 2. The circulating air was collected at 1 h-intervals until 12 h and at 24 h after administration, and the $H_2$ concentration in the circulating air was measured. ○ Administration of sucrose alone to conventional rats ($n=5$); ● administration of sucrose and MLE to conventional rats ($n=5$); ◇ administration of sucrose and MLE to antibiotic-treated rats ($n=5$). MLE, Morus alba leaf extract. Data are expressed as the mean±S.E. ($n=5$). * Significant differences between conventional rats given [U-$^{14}$C]-sucrose with and without MLE were evaluated at each time point at $p<0.05$ by repeated measures two-way ANOVA and Student $t$-test, respectively.]
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Recovery of $^{14}$C-radioactivity was not significantly different among the three groups, with 80.4% recovered in conventional rats administered [U-$^{14}$C]-sucrose alone, 83.8% in conventional rats administered [U-$^{14}$C]-sucrose with MLE, and 81% in antibiotic-treated rats administered [U-$^{14}$C]-sucrose and MLE, respectively.

**DISCUSSION**

To investigate the metabolic fate of sucrose inhibited from the digestion by MLE, [U-$^{14}$C]-sucrose solutions with or without MLE were administered orally to conventional and antibiotic-treated rats, and the excretion of $^{14}$CO$_2$ and H$_2$ was measured for 24 h. The excretion of $^{14}$CO$_2$ and H$_2$ was markedly affected by gut microbiota.

Sucrose, which is ingested orally, is digested completely by small intestinal enzymes, and so it does not reach the large intestine, which is inhabited by gut microbiota. As a result, H$_2$ is not excreted from the body. In the simultaneous administration of sucrose and MLE to conventional rats, the serum glucose elevation was suppressed significantly in comparison with that of administration of sucrose alone (Fig. 1), but serum glucose concentration increased slightly (15 mg/dL) but significantly from basal levels at 30 and 60 min after administration (*p* < 0.05). These results indicate that a part of sucrose administered with MLE was digested and absorbed in the small intestine. Thus, it demonstrates that the amount of MLE used in the present experiments is insufficient and could not inhibit completely sucrose digestion.

To examine the effect of gut microbiota on the decomposition of sucrose inhibited from the digestion by MLE, we used rats that gut microbiota was depleted with antibiotics, similar to previous studies (21, 32). The gut microbiota detectable by Mitsuoka method was mostly depleted after this treatment. Obvious excretion of $^{14}$CO$_2$ following the administration of [U-$^{14}$C]-sucrose solution with MLE in the antibiotic-treated rats suggests that a part of sucrose was digested by small

<table>
<thead>
<tr>
<th>Samples</th>
<th>Conventional rats</th>
<th>Antibiotic-treated rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{14}$C-sucrose</td>
<td>$^{14}$C-sucrose + MLE</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>59.1±2.61</td>
<td>58.0±2.61</td>
</tr>
<tr>
<td>Feces</td>
<td>1.43±0.25</td>
<td>4.97±0.51*</td>
</tr>
<tr>
<td>Urine</td>
<td>5.34±0.34</td>
<td>7.08±0.65</td>
</tr>
<tr>
<td>Serum</td>
<td>0.61±0.03</td>
<td>0.60±0.03</td>
</tr>
<tr>
<td>Contents (stomach, small intestine)</td>
<td>1.70±0.25</td>
<td>1.39±0.09</td>
</tr>
<tr>
<td>Contents (cecum, colon, rectum)</td>
<td>0.59±2.61</td>
<td>2.37±0.54</td>
</tr>
<tr>
<td>Small intestinal mucosa</td>
<td>0.82±0.03</td>
<td>0.82±0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.09±0.00</td>
<td>0.08±0.00</td>
</tr>
<tr>
<td>Testis</td>
<td>0.17±0.06</td>
<td>0.09±0.04</td>
</tr>
<tr>
<td>Liver</td>
<td>7.75±1.69</td>
<td>5.54±1.23</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.24±0.03</td>
<td>0.20±0.00</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>1.01±0.07</td>
<td>0.59±0.02</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>0.04±0.00</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>Total recovery (%)</td>
<td>80.4±3.05</td>
<td>83.8±3.37</td>
</tr>
</tbody>
</table>

Data were expressed as the mean±S.E. (n=5). MLE, *Morus alba* leaf extract.

* Significant differences between conventional rats given [U-$^{14}$C]-sucrose with and without MLE were evaluated by Student *t*-test, at *p*<0.05.
intestinal enzymes and utilized as energy source, because the [U-14C]-sucrose that continues to the large intestine is not fermented in rats depleted gut microbiota. These results are not contradictory to the significant elevation of serum glucose level in the administration of sucrose solution with MLE. They also indicate that a part of sucrose administered simultaneously with MLE to conventional rats is digested and absorbed in the small intestine, and the remaining sucrose that travels to the large intestine is then fermented by gut microbiota. Thus, sucrose prevented from the digestion by MLE is metabolized finally to CO₂ to produce energy through gut microbiota and is not excreted into the feces.

In conventional rats administered simultaneously [U-14C]-sucrose and MLE, the cumulative 14CO₂ excretion over 24 h was approximately 60% of the radioactivity administered and was similar to that in rats given [U-14C]-sucrose alone, although with a delay of 2–3 h (Fig. 2-B). This time lag demonstrates the period that the sucrose inhibited from the digestion by MLE is transferred to the large intestine and fermented by gut microbiota. In our previous study using [U-14C]-FOS the 24 h cumulative 14CO₂ excretion was approximately 60% with a time lag of 5–6 h relative to conventional rats given [U-14C]-sucrose alone (21). The difference in the delay (approximately 3 h) may suggest that a part of [U-14C]-sucrose administered simultaneously with MLE is digested and absorbed in the small intestine, and the remaining undigested [U-14C]-sucrose is fermented by gut microbiota. It seems that the difference of time lag of 14CO₂ excretion becomes smaller, if sucrose digestion in the small intestine is completely or more strongly inhibited by MLE.

The cumulative 14CO₂ excretion for 24 h was approximately 60% of the radioactivity administered in conventional rats given [U-14C]-sucrose solution with MLE (Fig. 2-B), and total recovery rate of 14C-radioactivity is 80–84% of the radioactivity administered. About 20% of the radioactivity administered appears to remain in the rat body as muscle and skin. If the experimental period is longer than 24 h, total recovery rate of 14C-radioactivity should be increased. 14CO₂ expelled by conventional rats given [U-14C]-sucrose solution with MLE contains both 14CO₂ produced from the oxidation of [U-14C]-sucrose digested and absorbed in the small intestine and 14CO₂ produced directly and from SCFAs through fermentation by gut microbiota in the large intestine. However, the 14CO₂ which is produced in the simultaneous administration of [U-14C]-sucrose and MLE to antibiotic-treated rats, is derived only from [U-14C]-sucrose digested in the small intestine, because rats without gut microbiota cannot produce CO₂ from the undigested [U-14C]-sucrose. Therefore, this indicates that the ratio of sucrose to MLE used in the present experiment allowed about 40% of the sucrose administered to be digested by small intestinal enzymes. And approximately 20% of radioactivity administered indicated that sucrose inhibited by the MLE was fermented by gut microbiota.

FOS and GOS are spontaneously fermented by gut microbiota in the lower intestine and is metabolized to SCFA, CO₂, H₂, and other products (12–15). A portion of the [U-14C]-sucrose administered simultaneously with MLE to conventional rats continued to the large intestine without being digested and was fermented by gut microbiota, and H₂ excretion was observed clearly. It has been shown that H₂ is produced during the fermentation of monosaccharides by gut microbiota (40). Acarbose treatment can increase the content of gut bifidobacterium in type 2 diabetes mellitus patients (41). The amount of 14C-radioactivity recovered in the lower intestine and feces was significantly higher in rats given both [U-14C]-sucrose and MLE as compared with rats given [U-14C]-sucrose alone (Table 1). Sucrose inhibited from the digestion by MLE may create a similar prebiotic effect to FOS.

The 14C radioactivity recovered in feces over 24 h was 1.43% in conventional rats administered [U-14C]-sucrose alone and 4.97% in conventional rats administering [U-14C]-sucrose with MLE. In conventional rats, most of the [U-14C]-sucrose administered orally was digested and absorbed in the small intestine, and so [U-14C]-sucrose cannot make it to the large intestine. Therefore, the radioactivity in the feces is expected to be the metabolites of sucrose secreted from the gastrointestinal tract. In addition, more 14C-radioactivity was recovered in the feces of conventional rats that were given [U-14C]-sucrose solution with MLE as compared with rats receiving [U-14C]-sucrose alone. In this case,
feces may contain a little product such as volatile acids and gut microbiota utilized [U-14C]-sucrose.

The 14C-radioactivity excreted into urine over 24 h was 4–7% of the total radioactivity administered in the three groups. Since sucrose is not absorbed intact in the small intestine, the radioactivity in urine seem to be metabolites of [U-14C]-sucrose containing urea, creatinine, and uric acid. Similar values have been obtained from other experiments using 14C-sugar substitutes (21, 30, 31). The 14C-radioactivity recovered in the contents of the lower intestine during the 24 h after administration was markedly high (approximately 21%) in antibiotic-treated rats administered [U-14C]-sucrose with MLE. The lower intestine of cecum and colon appears to contain the intact [U-14C]-sucrose prevented from the digestion by MLE.

To investigate the simultaneous excretion of 14CO2 and H2 produced during carbohydrate fermentation by gut microbiota, the cumulative excretion of H2 in Fig. 5 was calculated from the data in Fig. 3. The cumulative excretion of 14CO2 in Fig. 2 contains both 14CO2 produced by gut microbiota in the large intestine and 14CO2 after [U-14C]-sucrose was digested and absorbed in the small intestine and produced through oxidation in the body. To calculate the net cumulative excretion of 14CO2 produced by gut microbiota, each value in Fig. 5 was calculated as a percentage of each value from conventional and antibiotic-treated rats given [U-14C]-sucrose solutions with MLE. Thus, each point is the value from the conventional rats subtracted by the value from antibiotic-treated rats. The difference indicates the 14CO2 produced by gut microbiota during fermentation. Both time-dependent lines of cumulative 14CO2 and H2 excretion are similar and correspond well. These results show that 14CO2 and H2 are produced simultaneously in fermentation by gut microbiota and are discharged at the same time. Furthermore, it demonstrates that H2 excretion could be used directly to indicate the degree of fermentation of nondigestible carbohydrates.

In conclusion, we confirmed that [U-14C]-sucrose inhibited from digestion using MLE is metabolized spontaneously to SCFAs, CO2, H2, and other products by gut microbiota, similar to the degradation of nondigestible oligosaccharide as FOS. These results suggest that MLE suppresses the blood glucose elevation following sucrose and carbohydrates ingestions and is possible to prevent a lifestyle-related disease. In addition, we demonstrated that H2 and CO2 produced by gut microbiota are simultaneously excreted from the body and H2 becomes an indicator of fermentability of nondigestible carbohydrate.

Authorship

T.O. designed the study and had primary responsibility for the final content; T.O., T.T., and S.N. conducted research, analyzed data, and performed statistical analysis. K.T. and H.M. conducted research and analyzed the data. All authors have read and approved the final manuscript.

Disclosure of state of COI

The authors declare that they have no conflicts of interest.

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