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Running title: Effect of Maltobionic Acid on Bowel Movements in Healthy Subjects

Full title: *In Vitro* Utilization Characteristics of Maltobionic Acid and its Effects on Bowel Movements in Healthy Subjects

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Abbreviations
BDHQ, brief-type self-administered diet history questionnaire; BMI, Body Mass Index; CAS-MT, Constipation Assessment Scale Middle Term version; HPAEC-PAD, High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection; OD, Optical Density; SD, standard deviation; $T_g$, glass transition temperature.
**Abstract**

We examined the *in vitro* digestibility of maltobionic acid, obtained from enzymatic oxidation of maltose, its utilization by intestinal bacteria, and its biological effects on the bowel movements in healthy subjects. We found that maltobionic acid is not digested *in vitro* by saliva, gastric juice, or pancreatic juice. Moreover, it is digested only to a small extent by small intestinal enzymes. Among the 24 strains of intestinal bacteria, maltobionic acid was selectively utilized by *Bifidobacterium dentium* and *Bi. adolescentis*. We also evaluated the influence of long-term ingestion of maltobionic acid calcium salt on bowel movements in healthy Japanese women by a randomized, double-blind, placebo-controlled, crossover trial. Thirty-four subjects completed the study, and no adverse events related to the test food were observed. Ten subjects were excluded prior to the efficacy analysis because of conflict with the control criteria; the remaining 24 subjects were analyzed. Intake of test food containing 4 g maltobionic acid for 4 weeks caused a significant increase in the stool frequency, significant improvement in stool form scale and CAS-MT total scores as compared with the placebo group. These results suggest that maltobionic acid is an indigestible carbohydrate and is a promising therapeutic agent for improving the intestinal environment.

Keywords: maltobionic acid, indigestible saccharide, intestinal bacteria, bowel movement

**INTRODUCTION**

Ingestion of dietary fiber and oligosaccharides is thought to help improve intestinal function by affecting the regulation of gastrointestinal function (rate of gut movement of contents, digestion of nutrients, absorption, etc.) through physical and chemical interactions with the gastrointestinal tract, and the regulation of the internal environment (pH, water content, gut microbiota) in the gut lumen, etc.\(^1\)\(^2\)

Maltobionic acid, a sugar acid, has a structure in which glucose and gluconic acid are linked by α-1,4-glycosidic linkages and is characterized by an extremely weak sweet taste, a mildly sour taste, and forming stable salts with inorganic cations. The calcium salt of maltobionic acid, because of its
high water solubility, promotes calcium absorption and has a bone density-improving effect.\(^3\)\(^3\)\(^5\) It also has the advantage of having less adverse effects on taste and physical properties when formulated into foods because it has an extremely weak calcium-specific bitterness, a high glass transition temperature \(T_g\) (anhdyrous \(T_g = 145 \, ^\circ C\)), and a non-crystallizing nature even under high relative humidity conditions.\(^6\) While in addition to its use in mineral supplements and acid coagulant applications, gluconic acid, which is a component of maltobionic acid, has been reported to promote the growth of \textit{Bifidobacterium},\(^7\) no report on the digestibility of maltobionic acid and its utilization by intestinal bacteria exists. Therefore, in this study, we carried out a comparative \textit{in vitro} study of digestibility by intestinal enzymes and assimilation by intestinal bacteria of maltobionic acid and other oligosaccharides; we also evaluated their effects on fecal improvement in healthy human subjects.

**MATERIAL AND METHODS**

**Sample Preparation.** The analytical grade reagents viz. maltose monohydrate, maltotriose, maltotetraose, maltopentaose, sodium hydrogen carbonate, catalase, and glucose oxidase required for the synthesis of the sodium salts of maltobionic acid, maltotrionic acid, maltotetraonic acid and maltopentaonic acid were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan) and/or Amano Enzyme Inc. (Aichi, Japan), unless mentioned otherwise. The sodium salt of maltobionic acid was synthesized by adding 27 U of glucose oxidase, 1,800 U of catalase and 2.2 g of sodium hydrogen carbonate to a 30 % (w/v) maltose solution (30 mL) in a 100 mL baffled flask. The mixture was shaken at 300 rpm at 35 °C to facilitate the oxidation reaction. The synthesized maltobionic acid sodium salt was purified by treating with activated carbon at 80 °C for 30 min. Subsequently, the active carbon and enzymes were separated using vacuum filtration through Whatman glass microfiber filters (Grade GF/F) and membrane filters with a mixed cellulose ester pore size of 0.2 μm (Whatman, International Ltd., England). The sodium salts of the other sugars viz. maltotrionic acid, maltotetraonic acid, and maltopentaonic acid were synthesized by oxidizing maltotriose, maltotetraose, and maltopentaose (substrate concentrations of 3 %) under similar conditions.
A sample of corn syrup solids containing maltobionic acid (SourOligo C) was manufactured by San-Ei Sucrochemical Co., Ltd. The manufactured corn syrup solids sample contained 94.7 % solids, (4.4 % calcium, 60.3 % maltobionic acid, 16.7 % maltotronic acid, and 13.3 % other carbohydrates).

**In vitro digestibility of sugars.** In vitro digestibility of sugars, including maltose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), maltitol (FUJIFILM Wako Pure Chemical Corporation) and the sodium salts of maltobionic acid, maltotronic acid, maltotetraonic acid, and maltopentaonic acid was investigated according to Okada et al. with modifications. The in vitro digestion tests were performed with sodium salts of the sugar acids since use of sugar acids raised concerns about the effect on pH in the reaction solution. Analytical methods are as follows:

1) Hydrolysis with artificial human saliva was performed by using α-amylase from human saliva (type IX-A, Sigma-Aldrich Co. LLC). Artificial human saliva (1 mL, 40 U/mL α-amylase, 1 mM CaCl₂ and 50 mM Tris-maleate buffer (pH 6.0)) was added to 5 mL of sample solution (10 % (w/v) sugar, 1 mM CaCl₂, and 50 mM Tris-maleate buffer (pH 6.0)), incubated at 37 °C for 30 min and heated at 100 °C for 5 min to stop the reaction. One unit of α-amylase liberates 1.0 μmol of glucose from 0.1 % (w/v) soluble starch in 1 min at pH 6.9 at 37 °C.

2) Hydrolysis with artificial gastric juice was performed with an HCl-KCl buffer (pH 2.0). HCl-KCl buffer (2 mL, 50 mM, and pH 2.0) was added to 4 mL of sample solution (2.2 % (w/v) sugar), incubated at 37 °C for 100 min, and neutralized with 10 mM NaOH to stop the reaction.

3) Hydrolysis with pancreatic juice was performed with α-amylase from porcine pancreas (type I-A, Sigma-Aldrich). Pancreatic juice (0.5 mL; 20 U/mL of α-amylase) was added to 5 mL of sample solution (1 % (w/v) sugar, 1 mM CaCl₂, 50 mM Tris-maleate buffer (pH 6.6)), incubated at 37 °C for 6 h, and heated at 100 °C for 5 min to stop the reaction. The unit of enzyme activity defined was similar to that of α-amylase from human saliva.

4) Hydrolysis with small intestinal enzymes was performed with intestinal acetone powders from rat (Sigma-Aldrich). Intestinal enzyme solution (1 mL; 3.8 U/mL) was added to 5 mL of sample solution (1 % (w/w) sugar, 50 mM Tris-maleate buffer (pH 6.6)), incubated at 37 °C for 3 h, and heated at 100 °C for 5 min to stop the reaction. One unit of intestinal enzymes liberates 1.0 μmol
glucose from 1.0 % (w/v) maltose in 1 min at pH 6.0 at 37 °C.

Sugar composition was analyzed at the end of each reaction using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). HPAEC-PAD was performed on a DX-500 Carbohydrate system (Dionex, Sunnyvale, CA) equipped with a Dionex CarboPac PA-1 (250 × 4.0 mm) in combination with a CarboPac PA-1 guard column (50 × 4.0 mm); the column was eluted at 30 °C with 100 mM NaOH at flow rate of 1.0 mL/min. The sugars were analyzed by using a gradient of sodium acetate in 100 mM NaOH as follows: 0-5 min, 0 mM; 5-35 min, 0-200 mM. Glycerol was used as an internal control. The digestibility was assessed by calculating the peak area of each substrate using the following formula: Digestibility (%) = {[(Ia / Ib)Sa-Sb] / (Ia / Ib)Sa}×100. Peak areas are represented by Ia (internal standard sample before treatment), Ib (internal standard sample after treatment), Sa (sample before treatment), and Sb (test sample after treatment).

Utilization of sugars by intestinal bacteria in vitro. Bacteria were provided by the RIKEN BioResource Research Center (Ibaraki, Japan). The carbohydrates tested were maltobionic acid sodium salt, maltitol, glucose (San-ei Sucrochemical Co., Ltd., Chita, Japan), gluconic acid sodium salt (Fuso Chemical Co., Ltd., Osaka, Japan). The use of maltobionic acid in the intestinal bacteria utilization tests raised concerns about the effect on pH of the reaction solution, so the tests were performed using maltobionic acid sodium salt.

In vitro utilization of sugars by intestinal bacteria was investigated according to Okazaki et al.9) with modifications. Briefly, the bacterial strains were grown anaerobically on a BL agar plate at 37 °C by gas pack method (Anaero Pack; Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) and subsequently inoculated into GAM broth and maintained under anaerobic conditions overnight. The test medium was modified PYF medium containing 1.0 % (w/v) polypeptone (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 0.5 % (w/v) meat extract (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan), 0.5 % (w/v) yeast extract (Asahi Group Foods, Ltd., Tokyo, Japan), 0.3 % (w/v) K₂HPO₄ (Kanto Chemical Co., Inc., Tokyo, Japan), 0.1 % (w/v) Tween80 (Kanto Chemical Co., Inc.), 0.5 % (w/v) of the respective test carbohydrate was added to the modified PYF after sterilization, and solutions of sodium ascorbate (Kanto Chemical Co., Inc.) and L-cysteine-HCl (FUJIFILM Wako Pure
Chemical Corporation) were aseptically added to attain a final concentration of 1.0 and 0.05 %, respectively.

The test was conducted by adding 10 μL culture inoculum to 2 mL of the test medium and incubated anaerobically at 37 °C for 3 days. After incubation, the optical density (OD) at 660 nm and pH of the medium were measured.

The efficiency of carbohydrate utilization by each bacterial strain was evaluated quantitatively by calculating the difference in OD at 660 nm between the test medium and the control medium (without carbohydrate). The difference in OD was scored in the following manner: −, ΔOD<0.2; ±, 0.2≤ΔOD<0.4; +, 0.4≤ΔOD<0.6; ++, 0.6≤ΔOD<1.0; +++, 1.0≤ΔOD.

Effects of maltobionic acid on human bowel movements.

1. Study design and participants. A randomized, double-blind, placebo-controlled, crossover trial was performed. The study participants, Japanese adult women who worked at Chubu University (Aichi, Japan), were publicly recruited. A preliminary questionnaire was administered to those who gave written informed consent confirming their wish to participate in the study. Constipation prone persons aged 40 to 69 years or younger with a mean frequency of bowel movements of about 3 to 5 times per week were enrolled in the study. Among them, those who did not meet the following exclusion criteria were selected for participation in the study: (a) A medical history of malignant tumor, heart failure, or myocardial infarction; (b) Currently undergoing treatment for any of the following chronic disease: cardiac arrhythmia, hepatic disorder, renal disorder, cerebrovascular disorder, rheumatism, and diabetes mellitus; and (c) Subjects who were allergic to the test food-related products.

This study’s protocol received approval from the Chubu University Certified Review Board on September 21, 2018 (no. 300019-2). The study was conducted with full consideration to medical ethics and in accordance with the Declaration of Helsinki (2013) and the Ethical Guidelines for Medical and Health Research Involving Human Subjects. Testing was mainly conducted by the Chubu University. This study was registered with the University Hospital Medical Information Network (no. UMIN000034257).
2. Selection, randomization, and blinding. Thirty-five individuals were selected from 68 participants who agreed to participate in the study and assigned to two groups: 18 individuals in the test food antecedent group and 17 individuals in the control food antecedent group so that they did not differ greatly in age or BMI. Group allotments were conducted by an intermediary study controller using StatLight #11 (Yukms Co., Ltd., Kawasaki, Japan).

3. Test food. The test food used in the intervention was corn syrup solids containing maltobionic acid (SourOligo C, San-Ei Sucrochemical Co., Ltd.) powder packaged in stick-shaped packet form (4 g/packet). Corn syrup solids containing maltobionic acid contain 94.7 % solids, 4.4 % calcium, 60.3 % maltobionic acid, and 30.0 % other carbohydrates. The placebo food was a mix of 89.0 % crystalline maltose hydrate (Hayashibara Co., Ltd., Okayama, Japan) and 11.0 % calcium carbonate (Sankyo Seifun Co., Ltd., Okayama, Japan) contained in stick-shaped packets (4 g/packet). Its calcium component was 176 mg out of 4 g, the same as the test food. Both foods were in powder form and were in stick-shaped packets. Prior to the start of the study, the institutional review board confirmed that the foods could not be distinguished based on odor or color. The study participants ingested one packet (4 g) per day after meals with water or warm water. Study schedules included Intake period I (4 weeks), Washout Period (2 weeks), and Intake Period II (4 weeks).

4. Bowel movement questionnaire. The Japanese version of Constipation Assessment Scale Middle Term version (CAS-MT) \(^{10(11)12}\) and Bristol stool scale \(^{13(14)15}\) and, bowel movement diary were used to assess the subjective symptoms of the study participants. The fecal amount was assessed with reference to the size of the eggs.

CAS-MT was assessed on a 3-point scale of 0-2 for each of the eight constipation items, and the higher the score, the more likely it was to be constipation. The items were "Abdominal distention or bloating", "Change in amount of gas passed rectally", "Less frequent bowel movements", "Rectal fullness or pressure", "Rectal pain with bowel movement", "Small stool size", "Urge but inability to pass stool", and "Oozing liquid stool" the total score was assessed as the CAS score (range from 0 to 16).

The Bristol stool scale classified the stool form into seven categories: "Separate hard lumps, like nuts", "Lumpy soft stool", "Soft stool", "Pasty stool", "Liquid stool", "Mucus in stool", and "No stool passed". The stool consistency was assessed daily for the duration of the study.
"Sausage-shaped, but lumpy", "Like a sausage but with cracks on its surface", "Like a sausage or snake, smooth and soft", "Soft blobs with clear cut edges", "Fluffy pieces with ragged edges, a mushy stool", and "Watery, no solid pieces, entirely liquid", and each participant selected the corresponding one.

The bowel diary described the presence of bowel movements and the status of the bowel movements in terms of bowel movements by all study participants from the start to the end of the study.

5. **Diet survey.** We conducted a questionnaire survey of the participants’ nutritional intake for pre-ingestion and post-4W using a brief-type self-administered diet history questionnaire (BDHQ).

6. **Statistical analysis.** All outcomes were presented as mean ± SD. The primary outcome, the defecation days, was tested for carryover and aging effects, thereby confirming that the crossover design was adequate. Defecation days and fecal amount and CAS score were examined using participant testing at the pre-ingestion, post-2W, and post-4W time points, and intra- and inter-group comparisons were made. The intra-group comparisons were performed using Dunnett’s test with the time points and study participants as the fixed factors using the measured values in the following comparisons: pre-ingestion vs. post-2W and pre-ingestion vs. post-4W. Inter-group comparisons were performed by comparing the measured values and amount of change at all time points in the Test food versus Placebo group. The amount of change was determined by subtracting the pre-ingestion measured value from the post-2W or post-4W measured value. The pre-ingestion measured value and amount of change were analyzed using Student’s *t*-test, while the measured values for post-2W and post-4W were subjected to an inter-group comparison using analysis of covariance with a covariant as pre-ingestion. For Bristol stool scale, 'Improvement' was used when approaching 'Like a sausage or snake, smooth and soft,' and 'No improvement' was used when no change or far away, and the Wilcoxon signed-rank test was performed.

All statistical analyses were performed using two-sided testing, and the standard of significance was set at 5 %. The software used was Microsoft Excel 2010 and BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). Redundancy with other time points or other items was given no consideration.
RESULTS

In vitro digestibility of sugars. The digestion of maltobionic acid by artificial digestive juices was investigated in vitro (Table 1) as, to function as a prebiotic, a substance must reach the large intestine without digestion. Maltose, used as a control sugar, was hardly digested by the artificial gastric juice, but approximately 80% by the small intestinal enzymes. Maltitol was digested approximately 4% by the small intestinal enzymes but remained undigested in the presence of artificial saliva, artificial gastric juice, and artificial pancreatic juice. Similarly, maltobionic acid was not digested in artificial saliva, artificial gastric juice, or artificial pancreatic juice, and only slightly digested, approximately 3%, by small intestinal mucosal enzymes. Maltotronic acid was digested to a larger extent by small intestinal mucosal enzymes and decomposed into glucose and maltobionic acid. It was also confirmed that maltopentaonic acid was digested by artificial saliva and degraded to maltotriose and maltobionic acid.

Utilization of sugars by intestinal bacteria in vitro. Table 2 shows the results of evaluating the growth activity of intestinal bacteria in vitro in the presence of maltobionic acid. The monosugar acid, gluconic acid, was to a large extent selectively utilized by Bi. adolescentis, Bi. catenulatum, Bi. pseudocatenulatum, Bi. dentium, L. acidophilus, Ba. vulgatus, Eu. aerofaciens, Eu. limosum, C. butyricum, and C. difficile among the test strains. However, maltobionic acid was heavily utilized by Bi. dentium, Bi. adolescentis, C. ramosum, and C. coccoides, and to a lesser extent by Bi. infantis, Bi. longum, Bi. pseudocatenulatum, Bi. gallicum, L. acidophilus, L. gasseri, and C. butyricum, but not by Bacteroides or Eubacterium. More bacterial species utilized maltose and maltitol than maltobionic acid.

Effects of maltobionic acid on human bowel movements. Figure 1 shows the follow-up flow chart of the study participants. One of 35 study participants withdrew from the study citing personal reasons, with 34 completing the study. After analysis at the case review meeting, 24 subjects were included in the final analysis; we excluded one dropout from the study and ten for noncompliance (lack of entry in a diary, n=7; and test food or placebo consumption rates of 80% or less, n=3). ANOVA using
generalized linear models carried out using stool frequency as an indicator, group and food as fixed factors, and measurement of each study participant as a variable factor revealed no timing or ordinal effect. Thus, the use of a crossover study was validated.

Characteristics and dietary survey (BDHQ) data of the 24 subjects included in the analysis are shown in Tables 3 and 4, respectively; and the efficacy endpoints, i.e., defecation days, fecal amount, CAS score, and Bristol stool scale results, are shown in Tables 5, 6, 7, respectively. CAS score is summarized in Figure 2. The BDHQ results confirmed that there were no significant differences in the amount of dietary fiber consumed between the placebo- and test-food groups (Table 4). However, there was a significant increase in the change in the number of days of defecation from Pre-ingestion to post-4W intake in both the intra-group and inter-group comparisons at the time of intake of the trial food (Table 5). In addition, there was a significant decrease in the change in the CAS score from Pre-ingestion to post-4W after intake in both the intra-group comparison and the inter-group comparison at the time of intake of the test food (Figure 2 and Table 5). Among the CAS score items, significant improvement was observed in 3 items, "Less frequent bowel movements," "Small stool size," and "Urge but inability to pass stool." Bristol stool scale was significantly improved when the test food was consumed (Table 7).

**DISCUSSION**

Sugar acids, such as gluconic acid, lactobionic acid and maltobionic acid, are prepared by enzymatic, microbiological or noble metal catalytic oxidation. Maltobionic acid (4-O-α-D-Glucopyranosyl-D-gluconic acid), a molecule in which glucose is α-1,4-bonded to gluconic acid, is an indigestible disaccharide present in honey. Because this compound contains multiple hydroxyl groups and one carboxyl group, it possesses properties characteristic of both saccharides and acids. Maltobionic acid can also form a stable salt with inorganic cations, and when bound to Ca to form calcium salt of maltobionic acid, manifests high solubility in water as compared to existing Ca-containing compounds. Our previous animal studies on calcium absorption revealed an increase in the weight of cecal contents and an increase in short-chain fatty acids such as acetic acid, propionic
acid, and n-butyric acid with a 4-week long intake of the calcium salt of maltobionic acid.\textsuperscript{3,4)} However, it was not clear whether the effects observed in the animal study were due to maltobionic acid or gluconic acid. Therefore, in this study, by studying the digestibility of maltobionic acid, its utilization by intestinal bacteria, and its effects on the human intestines, we have tried to elucidate the role of intact maltobionic acid in the human body.

\textit{In vitro} digestibility studies confirmed that maltobionic acid is an indigestible oligosaccharide with digestion tolerance comparable to that of maltitol. In addition, since maltotrionic acid is mostly decomposed into maltobionic acid and glucose by digestive enzymes, maltobionic acid was speculated to work as a functional ingredient \textit{in vivo}.

In an intervention study in constipation-prone individuals, defecation days slightly increased in both the placebo and test food groups at week 2, but remained unchanged in the placebo group at week 4, whereas a further increase in defecation days was observed in the test food group. Therefore, the increase in defecation days in the placebo group at week 2 is due to the placebo effect, since no improvement in the subjective symptoms, according to the CAS score, was observed in the placebo group prior to week 4 of ingestion. In the test food group, the subjective symptom of constipation, such as a change in stool hardness to moderate softness, showed an improving tendency on the subjective symptom by the CAS score from week 2 to week 4. Further, the property necessary for the improvement of the intestinal function by the continuous intake of the maltobionic acid as well as the existing oligosaccharide was possessed. The results of the Dietary Survey (BDHQ) confirmed that there were no significant differences between the placebo- and test-food groups in terms of dietary fiber content, indicating that there was no effect of dietary fiber consumption on bowel movements. Based on these results, we believe that the increase in short-chain fatty acids in previous animal studies\textsuperscript{3,4)} and the results in our human study support the effect of maltobionic acid rather than gluconic acid.

Human-residential bifidobacteria (HRB), bifidobacteria detected mainly in the human intestine, number about 10 species, and the species inhabiting infants and adults are also different.\textsuperscript{20)} Xylooligosaccharides,\textsuperscript{9)} galactooligosaccharides,\textsuperscript{21,22)} and fructooligosaccharides\textsuperscript{23)} have been reported to be
selectively utilized by many species of bifidobacteria and lactobacilli, notably \textit{Bi. longum} which predominates in a wide range of ages from infants to adults.\textsuperscript{24)}

The digestibility of maltobionic acid \textit{in vitro} was only slightly utilized by \textit{Bi. longum}, but was confirmed to have strong proliferative activity in the second most predominant HRB, \textit{Bi. adolescentis}, and \textit{Bi. dentium}. Gluconic acid was used for \textit{Bi. catenulatum} and \textit{Bi. pseudocatenulatum} as well as \textit{Bi. adolescentis} and \textit{Bi. dentium}. These are also the predominant HRB in the adult intestine, suggesting that these HRB have a mechanism for selective utilization of sugars with a gluconate backbone. \textit{Bi. adolescentis} and \textit{Bi. dentium}, which have proliferative activity \textit{in vitro}, have been reported to be highly capable of folate biosynthesis.\textsuperscript{25)} Folate plays an important role in cellular metabolisms such as in nucleic acid metabolism and DNA methylation,\textsuperscript{26)} and the activation of folate production by HRB by ingestion of maltobionic acid seems to play an important role in the maintenance of cellular activity. \textit{Bi. dentium} has also been reported to have $\gamma$-aminobutyric acid (GABA) production activity.\textsuperscript{27)} Since GABA is an inhibitory neurotransmitter and has been reported to have a hypotensive effect and a diuretic effect, it is expected to improve bioregulatory function by increasing the activity of these HRBs through ingestion of maltobionic acid.

Galactooligosaccharides and fructooligosaccharides have been reported to be used by Bacteroides, Clostridium, and Eubacterium. However, maltobionic acid is not used by Bacteroides and Eubacterium. It is only slightly utilized by Clostridium, a butyric acid-producing bacterium, so compared with existing oligosaccharides it can be digested by few bacteria. Therefore, the effect of improving bowel movements in the human study may have been slow to affect the intestinal bacterial flora due to the small number of intestinal bacteria which can metabolize maltobionic acid, and it may have taken 4 weeks to obtain a significant difference. Of the 8 CAS scores, significant decreases were observed in 3 CAS scores, namely "Less frequent bowel movements," "Small stool size," and "Urge but inability to pass stool." Water-soluble dietary fiber is known to improve stool hardness and volume owing to osmotic water transfer to the large intestine and increased water retention\textsuperscript{28)(29)} and the calcium salt of maltobionic acid has high water retention properties; therefore, it is believed that the continuous intake of maltobionic acid increases the water content in feces, thereby making it
moderately soft, which facilitates defecation and improves the subjective symptoms of constipation. In the future, it is necessary to analyze information on effective intake and feces before and after intake of maltobionic acid by a next-generation sequencer, etc., and to examine the effect on the human intestinal bacterial flora in order to further clarify the mechanism on defecation improvement effect and bioregulation function of maltobionic acid.

AUTHOR CONTRIBUTIONS
Ken Fukami, Daiki Suehiro, and Motoko Onishi designed the research protocol. Ken Fukami wrote the manuscript. Daiki Suehiro and Motoko Ohnishi reviewed and edited the manuscript. Ken Fukami had primary responsibility for the final content. All authors read and approved the final version of the manuscript.

CONFLICTS OF INTEREST
Ken Fukami and Daiki Suehiro are employees of San-ei Sucrochemical Co., Ltd. which supplies a food product containing maltobionic acid.

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Figure Captions

**Figure 1.** Flow diagram of subject recruitment and retention throughout the course of the study.

**Figure 2.** Summarize of the results of the Japanese version of the Constipation Assessment Scale Middle Term version (CAS-MT).

*P < 0.05 (vs. Pre-ingestion).
Table 1. Hydrolysis rate (%) of maltobionic acid with artificial digestive juices \textit{in vitro}.

Digestibility of samples was assessed \textit{in vitro} at 37° C.

|                | Artificial saliva | Artificial digestive juices | Artificial pancreatic juice | Small intestinal mucosal juice |
|----------------|-------------------|-----------------------------|----------------------------|-----------------------------|
| Maltose        | 0.0               | 0.0                         | 0.0                        | 76.4                        |
| Maltitol       | 0.0               | 0.0                         | 0.0                        | 3.8                         |
| Maltobionic acid Na salt | 0.0       | 0.0                         | 0.0                        | 3.3                         |
| Maltotronic acid Na salt | 0.0       | 0.0                         | 0.0                        | 49.3                        |
| Maltotetraonic acid Na salt | 0.0      | 0.0                         | 0.0                        | 100.0                       |
| Maltoctaonic acid Na salt | 68.6      | 0.0                         | 100.0                      | 100.0                       |

Glycerol was used as the internal standard. Digestibility (\%) = \{[(Ia / Ib)Sa-Sb] / (Ia / Ib)Sa\} x 100.

Peak areas are represented by Ia (internal standard sample before treatment), Ib (internal standard sample after treatment), Sa (sample before treatment), and Sb (test sample after treatment).
Table 2. Utilization of maltobionic acid by intestinal bacteria in vitro.

| Origin                  | Glucose | Maltose | Maltitol | Gluconic acid Na salt | Maltobionic acid Na salt |
|-------------------------|---------|---------|----------|-----------------------|-------------------------|
| *Bifidobacterium*       |         |         |          |                       |                         |
| *adolescentis*          | JCM 1275 | +++     | +++      | +++                   | +                       |
| *breve*                 | JCM 1192 | +       | +        | -                     | -                       |
| *Infantis*              | JCM 1222 | +++     | +++      | ±                     | -                       |
| *longum*                | JCM 1217 | +++     | +++      | -                     | ±                       |
| *catenulatum*           | JCM 1194 | +++     | +++      | -                     | +                       |
| *bifidum*               | JCM 1255 | +       | +        | -                     | -                       |
| *psuedocatenulatum*     | JCM 1200 | +++     | +++      | ++                    | +                       |
| *dentium*               | JCM 1195 | +++     | +++      | ++                    | ++                      |
| *gallicum*              | JCM 8224 | +++     | +++      | -                     | ±                       |
| *angulatum*             | JCM 7096 | +++     | +++      | -                     | -                       |
| *Lactobacillus*         |         |         |          |                       |                         |
| *acidophilus*           | JCM 1132 | +++     | +++      | ±                     | +                       |
| *gasseri*               | JCM 1131 | +++     | +++      | ±                     | -                       |
| *salivarius*            | JCM 1231 | +++     | +++      | -                     | -                       |
| *Bacteroides*           |         |         |          |                       |                         |
| *vulgatus*              | JCM 5826 | +++     | +++      | ++                    | +                       |
| *ovatus*                | JCM 5824 | +       | +        | -                     | -                       |
| *thetaiotaomicron*      | JCM 5827 | ±       | ±        | ±                     | -                       |
| *Parabacteroides*       |         |         |          |                       |                         |
| *distasonis*            | JCM 5825 | ±       | ±        | ±                     | -                       |
| *Eubacterium*           |         |         |          |                       |                         |
| *aerofaciens*           | JCM 7790 | +++     | +++      | +++                   | +                       |
| *limosum*               | JCM 6421 | +++     | +++      | ++                    | +                       |
| *Clostridium*           |         |         |          |                       |                         |
| *butyricum*             | JCM 7840 | +++     | +++      | +++                   | +                       |
| *difficile*             | JCM 1296 | +       | -        | -                     | -                       |
| *paraputrificum*        | JCM 1293 | +++     | -        | -                     | -                       |
| *ramosum*               | JCM 1298 | +++     | +++      | -                     | +                       |
| *cocoides*              | JCM 1395 | +++     | +++      | -                     | +                       |

Growth activity of intestinal bacteria was assessed in vitro in the presence of maltobionic acid.

Growth ratings: ++++, ΔOD>1.0; ++, 1.0<ΔOD≤0.6; +, 0.6<ΔOD≤0.4; ±, 0.4<ΔOD≤0.2; −, 0.2<ΔOD; ΔOD = (test OD) - (control OD).

Table 3. Baseline age and anthropometric characteristics of healthy adolescent women analyzed in the maltobionic acid defecation improvement study.

|                           | Analytical subject population (n=24) |
|---------------------------|-------------------------------------|
| Age                       | 51.6 ± 5.7                          |
| Body height (cm)          | 160.2 ± 4.8                         |
| Body weight (kg)          | 53.8 ± 7.0                          |
| BMI (kg/m²)               | 21.0 ± 2.7                          |

Values are mean ± SD.
Table 4. Results of the diet survey of analyzed subjects using a brief-type self-administered diet history questionnaire (BDHQ).

| Item             | Ingested food | n   | Pre-ingestion | Post-4W    |
|------------------|---------------|-----|---------------|------------|
| Calories (kcal/day) | Test food     | 24  | 1549 ± 380    | 1388 ± 406 |
|                  | Placebo food  |     | 1493 ± 437    | 1414 ± 363 |
| Carbohydrate (g/day) | Test food    | 24  | 197 ± 51      | 177 ± 54   |
|                  | Placebo food  |     | 199 ± 66      | 183 ± 48   |
| Protein (g/day)   | Test food     | 24  | 61.2 ± 19.6   | 56.1 ± 19.5|
|                  | Placebo food  |     | 58.7 ± 20.1   | 58.0 ± 17.6|
| Fat (g/day)       | Test food     | 24  | 53.9 ± 14.1   | 48.0 ± 15.8|
|                  | Placebo food  |     | 48.5 ± 14.2   | 47.4 ± 14.4|
| Dietary Fiber (g/day) | Test food   | 24  | 10.1 ± 3.3    | 9.6 ± 3.3  |
|                  | Placebo food  |     | 10.2 ± 3.5    | 9.7 ± 2.9  |

A brief-type self-administered diet history questionnaire (BDHQ) was filled out by participants before the study and after 4 weeks. Values are mean ± SD.
Table 5. Results from the analysis of the bowel movement diary and the Japanese version of the Constipation Assessment Scale Middle Term version (CAS-MT) of healthy adolescent women who ingested maltobionic acid for 4 weeks.

| Item                                             | Ingested food | n  | Pre-ingestion | Post-2W | Post-4W | Amount of change |
|--------------------------------------------------|---------------|----|---------------|---------|---------|------------------|
| Defecation days (day/week)                       | Test food     | 24 | 4.54 ± 0.66   | 5.29 ± 1.90 | 5.88 ± 1.39 | 0.75 ± 1.59 |
|                                                  | Placebo food  |    | 4.79 ± 0.93   | 5.50 ± 1.29 | 5.46 ± 1.72 | 0.71 ± 0.91 |
| Fecal amount (piece/day)                         | Test food     | 24 | 2.19 ± 1.10   | 2.34 ± 1.08 | 2.47 ± 1.42 | 0.16 ± 0.65 |
|                                                  | Placebo food  |    | 2.24 ± 1.14   | 2.48 ± 1.25 | 2.36 ± 1.20 | 0.24 ± 0.66 |
| CAS score                                        | Test food     | 24 | 3.08 ± 2.57   | 2.54 ± 2.02 | 1.46 ± 1.59 | *-0.54 ± 2.36 |
|                                                  | Placebo food  |    | 3.17 ± 3.33   | 3.26 ± 2.38 | 3.30 ± 3.02 | 0.09 ± 3.48 |
| Abdominal distention or bloating                 | Test food     | 24 | 0.58 ± 0.65   | 0.54 ± 0.66 | 0.50 ± 0.59 | -0.04 ± 0.62 |
|                                                  | Placebo food  |    | 0.48 ± 0.73   | 0.65 ± 0.78 | 0.52 ± 0.59 | 0.17 ± 0.49 |
| Change in amount of gas passed rectally          | Test food     | 24 | 0.08 ± 0.28   | 0.17 ± 0.48 | 0.13 ± 0.34 | 0.08 ± 0.28 |
|                                                  | Placebo food  |    | 0.22 ± 0.60   | 0.30 ± 0.56 | 0.39 ± 0.66 | 0.09 ± 0.42 |
| Less frequent bowel movements                     | Test food     | 24 | 0.46 ± 0.66   | 0.21 ± 0.41 | 0.13 ± 0.34 | *-0.25 ± 0.68 |
|                                                  | Placebo food  |    | 0.43 ± 0.66   | 0.39 ± 0.50 | 0.48 ± 0.51 | -0.04 ± 0.71 |
| Rectal fullness or pressure                       | Test food     | 24 | 0.42 ± 0.58   | 0.38 ± 0.58 | 0.25 ± 0.44 | -0.04 ± 0.62 |
|                                                  | Placebo food  |    | 0.43 ± 0.66   | 0.57 ± 0.59 | 0.39 ± 0.50 | 0.13 ± 0.69 |
| Rectal pain with bowel movement                   | Test food     | 24 | 0.29 ± 0.62   | 0.25 ± 0.61 | 0.04 ± 0.20 | -0.04 ± 0.46 |
|                                                  | Placebo food  |    | 0.30 ± 0.56   | 0.17 ± 0.49 | 0.50 ± 0.63 | -0.13 ± 0.46 |
| Small stool size                                  | Test food     | 24 | 0.46 ± 0.59   | 0.33 ± 0.48 | 0.13 ± 0.34 | *-0.13 ± 0.61 |
|                                                  | Placebo food  |    | 0.48 ± 0.59   | 0.45 ± 0.51 | 0.57 ± 0.51 | -0.04 ± 0.47 |
| Urge but inability to pass stool                  | Test food     | 24 | 0.67 ± 0.82   | 0.63 ± 0.65 | 0.25 ± 0.44 | -0.04 ± 0.69 |
|                                                  | Placebo food  |    | 0.48 ± 0.67   | 0.61 ± 0.66 | 0.52 ± 0.67 | 0.13 ± 0.63 |
| Oozing liquid stool                               | Test food     | 24 | 0.13 ± 0.34   | 0.04 ± 0.20 | 0.04 ± 0.20 | -0.08 ± 0.28 |
|                                                  | Placebo food  |    | 0.04 ± 0.21   | 0.04 ± 0.21 | 0.04 ± 0.21 | 0.00 ± 0.00 |

The study food was evaluated in a crossover study in which one pack (4 g) per day was consumed for 4 weeks, and the washout times were 2 weeks apart. The CAS-MT includes eight items, each of which is self-rated by the participants as 'no problem' (score of 0), 'some problem' (score of 1), or 'severe problem' (score of 2). The item ratings are then summed, so the overall score may range from 0 (no constipation) to 16 (worst possible constipation). Values are mean ± SD. *$P < 0.05$ (vs. Pre-ingestion). $^a$P < 0.05 (vs. Placebo food).
Table 6. Serial changes in Bristol stool scale in healthy adolescent women who ingested maltobionic acid for 4 weeks.

| Ingested food | n  | Watery, no solid pieces, entirely liquid | Fluffy pieces with ragged edges, a mushy stool | Soft blobs with clear cut edges | Like a sausage or smooth, smooth and soft | Like a sausage but with cracks on its surface | Sausage-shaped, but lumpy | Separate hard lumps, like nuts |
|---------------|----|----------------------------------------|---------------------------------------------|-------------------------------|-------------------------------------------|---------------------------------------------|------------------------|----------------------------|
| Pre-ingestion | Test food | 24 | 0 | 0 | 2 | 8 | 5 | 7 | 2 |
|                | Placebo food |    | 0 | 1 | 2 | 9 | 7 | 3 | 2 |
| Post-2W        | Test food | 24 | 0 | 1 | 1 | 10 | 3 | 7 | 2 |
|                | Placebo food |    | 0 | 0 | 2 | 12 | 2 | 5 | 3 |
| Post-4W        | Test food | 24 | 0 | 0 | 2 | 13 | 6 | 2 | 1 |
|                | Placebo food |    | 0 | 1 | 2 | 8 | 4 | 5 | 4 |

Bristol stool scale scores for participants at 3 time points.

Table 7. Improvement in Bristol stool scale in healthy adolescent women who ingested maltobionic acid for 4 weeks.

| Ingested food | n  | Improvement | No improvement | p value |
|---------------|----|-------------|----------------|---------|
| Pre-ingestion | Test food | 24 | 5 | 19 | 0.220 |
|                | Placebo food |    | 2 | 22 |       |
| vs. Post-2W    | Test food | 24 | 11 | 13 | 0.011 |
|                | Placebo food |    | 3 | 21 |       |

Distribution of participants showing improvement or no improvement during study intervals.

Patient scores moving towards "Like a sausage or snake, smooth and soft" were defined as "Improvement," otherwise scores moving away or with no change were defined as "No improvement." The number of relevant participants is indicated.
Figure 1
Figure 2