

Treatment with broad-spectrum antibiotics upregulates SglT1 and induces small intestinal villous hyperplasia in mice

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Although extensive evidence indicates that the gut microbiota plays a crucial role in regulating glucose homeostasis, the exact regulatory mechanism remains unclear. This study aimed to investigate the effect of broad-spectrum antibiotics on the expression of glucose transporters, histomorphology of the small intestine, and glucose metabolism in mice. C57BL/6 mice were administered drinking water with or without a broad-spectrum antibiotic combination for 4 weeks. Thereafter, an oral glucose tolerance test was performed. Body weight, small intestine histopathology, mRNA levels of glucose transporters (SGLT1 and GLUT2) and intestinal transcription factors (CDX1 and CDX2) were evaluated. SGLT1 and CDX1 were upregulated in the small intestine upon antibiotic administration compared with that in the control group. The height and surface area of the jejunal villi were significantly higher upon antibiotic administration than in the control group. Fasting glucose levels were significantly higher upon antibiotic administration than in the control group. The present results indicate that treatment with broad-spectrum antibiotics upregulates SGLT1 and CDX1 and induces hyperplasia in the small intestine, thus increasing fasting blood glucose levels. Our results further the current understanding of the effects of broad-spectrum antibiotics on the gut microbiota and glucose homeostasis that may have future clinical implications.

Key Words: antibiotics, gut microbiota, SGLT1, intestinal hyperplasia, glucose tolerance

Antibiotics are widely used for treating and preventing pathogenic infections; however, they can cause dysbiosis and affect human health. Antibiotic-induced dysbiosis is a risk factor for obesity, metabolic disorders, and diabetes mellitus (DM).^(1,2) Treatment with a combination of broad-spectrum antibiotics in early life accelerates the onset of DM in adulthood in mice and humans.^(3–6) Changes in the abundance of *Akkermansia muciniphila*, *Lactobacillus* spp., *Clostridium* spp., and *Bacteroides* spp. have been reported in patients with diabetes and obesity.^(7–9) Moreover, probiotics potentially alter glucose homeostasis in mice and humans.^(10,11) Thus, the intestinal microbiota plays an important role in regulating glucose homeostasis. Despite advancements in the understanding of the basis of host–microbiome symbiosis, the exact mechanism through which the gut microbiota potentially regulates host glucose metabolism remains unclear.

In the small intestine, glucose absorption is mediated by sodium-dependent glucose transporter 1 (SGLT1) and facilitative glucose transporter 2 (GLUT2), which are associated with

incretin responses. SGLT1 is apically expressed and transports glucose from the intestinal lumen to the cytosol of enterocytes, whereas GLUT2 is basolaterally expressed and transports glucose from the cytosol of enterocytes to the blood.^(12–14) Intestinal SGLT1 is reportedly upregulated in diabetes and this glucose transporter has been speculated as a novel therapeutic target in diabetes.^(15–18) Although intestinal glucose absorption regulates glucose homeostasis and the gut microbiota is an important regulator of glucose homeostasis,⁽¹⁹⁾ the effects of antibiotic-induced dysbiosis on intestinal glucose transporters are unclear. Therefore, this study aimed to investigate the effect of broad-spectrum antibiotics on expression of the glucose transporters (SGLT1 and GLUT2), histomorphology of the small intestine, and glucose metabolism in mice.

Materials and Methods

Mice. A total of 24 seven-week-old C57BL/6 mice (Shimizu Laboratory Supplies, Kyoto, Japan) were acclimated for one week in the animal facility of the Division of Animal Experiment, Kyoto Prefectural University of Medicine in accordance with the guidelines for Institutional Animal Care, in plastic cages ($n = 5$ or 6 mice per cage) with *ad libitum* access to drinking water and a basal diet under controlled conditions of humidity, light (12/12-h light/dark) and temperature. All animal experiments and experiments were approved by the Experimental Animal Research Committee of the Kyoto Prefecture University of Medicine.

Segregation of mice and extraction of mouse tissue. After a week of acclimatization, the mice were segregated into two body-weight-matched groups ($n = 12$). Antibiotic-treated mice were treated with a combination of broad-spectrum antibiotics (1 g/L ampicillin, 500 mg/L vancomycin, and 1 g/L neomycin sulfate) in drinking water.⁽²⁰⁾ Food intake and body weights were recorded every day. Four weeks after antibiotic administration, all mice were euthanized via cervical dislocation. Cecum and epididymal adipose tissue were extracted and weighed and 2-cm jejunum segments approximately 4 cm distal to the ligament of Treitz were harvested. Jejunum tissue was divided into two parts, of which one was snap-frozen in liquid nitrogen and stored at -80°C until use, while the other part was fixed in 10% formaldehyde for 24 h, dehydrated, and embedded in paraffin.

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Oral glucose tolerance test (OGTT). Four weeks after antibiotic administration, OGTT was performed among all mice. After 16 h of fasting with *ad libitum* access to water, mice were orally administered 40% glucose with a feeding gavage (at 2 g/kg body weight), using a stainless-steel gavage needle. Blood samples were obtained from the tail vein before and after 30, 60, 90, and 120 min of glucose administration. Blood glucose was measured using a glucometer. Area under the curve (AUC) values for plasma glucose were determined.

Quantitative reverse transcription-PCR analysis. *Sglt1*, *Glut2*, *Cdx1*, and *Cdx2* expression levels were quantified via real-time PCR. Total RNA was isolated from homogenized mouse tissue, using the acid guanidinium phenol chloroform method with Isogen (Nippon Gene Co. Ltd., Tokyo, Japan) and stored at -80°C until use. Thereafter, 1 μg of extracted RNA was reverse-transcribed into first-strand cDNA, using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. Real-time PCR for *Sglt1*, *Glut2*, *Cdx1*, *Cdx2*, and *Actb* was carried out using the 7300 Real-time PCR system (Applied Biosystems) using the DNA-binding dye SYBR[®] Green to detect the PCR products. The following primers were used: *Sglt1*, sense 5'-CCTCTCGGCCAAGAACATGT-3' and antisense 5'-TTCAGATAGCCACACAGGGTACA-3'; *Glut2*, sense 5'-TGGAAGATCAAAGCAATGTTG-3' and antisense 5'-CATCAAGAGGGCTCCAGTCAA-3'; *Cdx1*, sense 5'-CGCCTAGAGCTGGAA AAGGA-3' and antisense 5'-TTAGCAGCCAGCTCGGACTT-3'; *Cdx2*, sense 5'-CGAGCCCTTGAGTCCTGTGA-3' and antisense 5'-AACCCAGGGACAGAACCA-3'; *Actb*, sense 5'-TATCCA CCTCCAGCAGATGT-3' and antisense 5'-AGCTCAGTAACA GTCCGACTA-3'.

Histopathological analysis. Small intestinal specimens were gently rinsed with normal saline, fixed in formalin solution, and embedded in paraffin. Sections were deparaffinized, hydrated, and stained with hematoxylin and eosin. Histopathological findings were evaluated using a microscope (BX50; Olympus, Tokyo, Japan) in a blinded manner. Villar height and width and crypt depth were measured for six mice and segments in each group. Villar surface area was determined as follows: $\pi \times \text{villar length} \times \text{villar width}$.⁽²¹⁾

Microbiome analysis. Microbiome analysis was performed according to the previous reports.^(22,23) Briefly, the QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) was used to extract genomic DNA from the collected fecal pellets. Microbiome analyses by 16S rRNA gene sequencing were conducted by Takara Bio Inc. DNA specimen from feces was amplified using a 16S (V3–V4) metagenomic library construction kit for NGS (Takara Bio Inc., Kusatsu, Japan) with primer pairs 341F (5'-TCGTCGCGCAGCGTCAGATGTGATAAGAGACAGCCTACG GGNGGCWGCAG-3') and 806R (5'-GTCTCGTGGGCTCGG AGATGTGATAAGAGACAGGGACTACHVGGGTWTCTAAT-3') corresponding to the V3–V4 region of the 16S rRNA gene. The amplicons were purified and prepared for the sequencing library by using AMPure XP beads (Beckman Coulter, Brea, CA). The purified amplicon library was sequenced on an Illumina Miseq platform (Illumina Inc., San Diego, CA) at the Biomedical Center at Takara Bio. The processing of sequence data, including chimera check, operational taxonomic unit (OTU) definition, and taxonomy assignment, was performed using QIIME 2,⁽²⁴⁾ DATA2,⁽²⁵⁾ and VSEARCH.⁽²⁶⁾

Statistical analysis. Data are expressed as mean \pm SE of the mean values. Differences between the antibiotic-treated and control groups were assessed via the Student's *t* test. Differences were considered significant at a two-sided *p* value <0.05 . All statistical analyses were performed using JMP 13 software (SAS Institute, Inc., Cary, NC).

Results

Effect of antibiotics on body weight and epididymal adipose tissue. On examining food intake per period, food intake decreased in the antibiotic-treated group compared with that in the control group in the initial period (days 1–7) ($p = 0.0002$); however, thereafter, no significant difference in food intake was observed between the two groups (Supplemental Fig. 1*). Body weight did not significantly differ between the two groups at the end of the experiment (Fig. 1A). However, the cecum weight was significantly greater in the antibiotic-treated group than in the control group ($p < 0.0001$), and consequently, the average body weight excluding the cecum weight was significantly lower in the antibiotic-treated group than in the control group ($p = 0.023$) (Fig. 1B–D). Epididymal adipose weight was significantly lower in the antibiotic-treated group than in the control group ($p = 0.035$) (Fig. 1E).

Effect of antibiotics on the expression of intestinal glucose transporters. SGLT1 expression levels in jejunal epithelial cells were significantly higher in the antibiotic-treated group than in the control group ($p = 0.009$, Fig. 2A); however, GLUT2 expression levels did not differ significantly between the two groups (Fig. 2B). Immunostaining showed that SGLT1 expressed mainly on the apical pole of epithelial cells continuously in antibiotics-treated mice (Fig. 2C).

Effect of antibiotics on the histopathological features of the intestinal mucosa. The jejunal villar height and surface area were significantly greater in the antibiotic-treated group than in the control group ($p < 0.001$), while crypt depth and villar width did not significantly differ. Regular villar morphology was retained in the antibiotic-treated group (Fig. 3A and B).

Furthermore, on analyzing CDX1/2 expression levels, both intestinal transcription factors, CDX1 mRNA levels were significantly higher in the antibiotic-treated group than in the control group ($p = 0.028$, Fig. 3C-1), whereas CDX2 mRNA levels did not significantly differ between the two groups (Fig. 3C-2).

OGTT. Fasting glucose levels were significantly higher in the antibiotic-treated group than in the control group ($p = 0.043$) (Fig. 4A). However, the OGTT revealed no significant differences in plasma glucose levels after a glucose load (30, 60, 90, and 120 min) between the two groups (Fig. 4A). Furthermore, the average AUC for plasma glucose did not differ between the two groups (Fig. 4B).

Gut microbiota diversity and composition. The effects of a broad-spectrum antibiotic combination on the intestinal microbiota were investigated. As for the diversity of gut microbiota using different alpha-diversity indices (the Shannon and the Chao 1 index), both the values in antibiotic-treated group were significantly higher than the control group ($p = 0.0049$ and $p = 0.0062$, respectively, Supplemental Fig. 2A*). A PCoA analysis using the weighted UniFrac suggested that overall community composition of the microbiota from the antibiotic-treated group was very different from the control group (Supplemental Fig. 2B*). Subsequently, the differences in the gut microbial structure in two groups were taxonomically evaluated at the phylum level. Compared to the control, antibiotic-treated group had a lower abundance of the phyla Bacteroidetes and Verrucomicrobia, and higher abundance of the phyla Proteobacteria (Fig. 5A). The Firmicutes/Bacteroidetes ratio was higher in the antibiotic-treated group than in the control group, though it did not reach statistical significance ($p = 0.11$, Fig. 5B).

Discussion

The gut microbiota has recently been recognized as an important regulator of glucose homeostasis. Previous reports indicate that there is a cause-effect relationship between the gut microbiota and insulin sensitivity, and several mechanisms

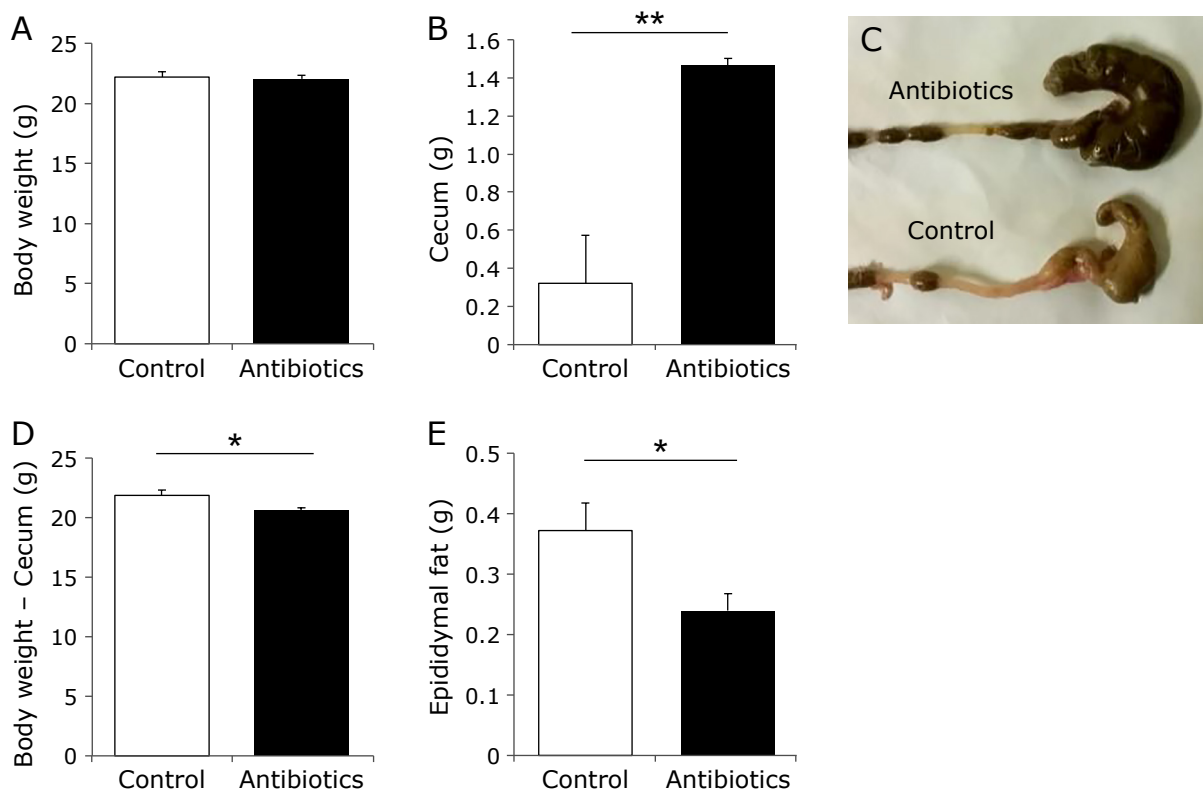


Fig. 1. Body, cecum and epididymal fat weight. (A) Body weight did not differ between groups. (B, C) Cecum weight was significantly higher in the antibiotic-treated group than in the control group. (D) Hence, the body weight, excluding the cecum weight, was significantly lower in the antibiotic-treated group. (E) The weight of epididymal fat was significantly lower in the antibiotic-treated group than in the control group. Values are expressed as mean with SE of six mice in each group. * $p < 0.05$, ** $p < 0.001$.

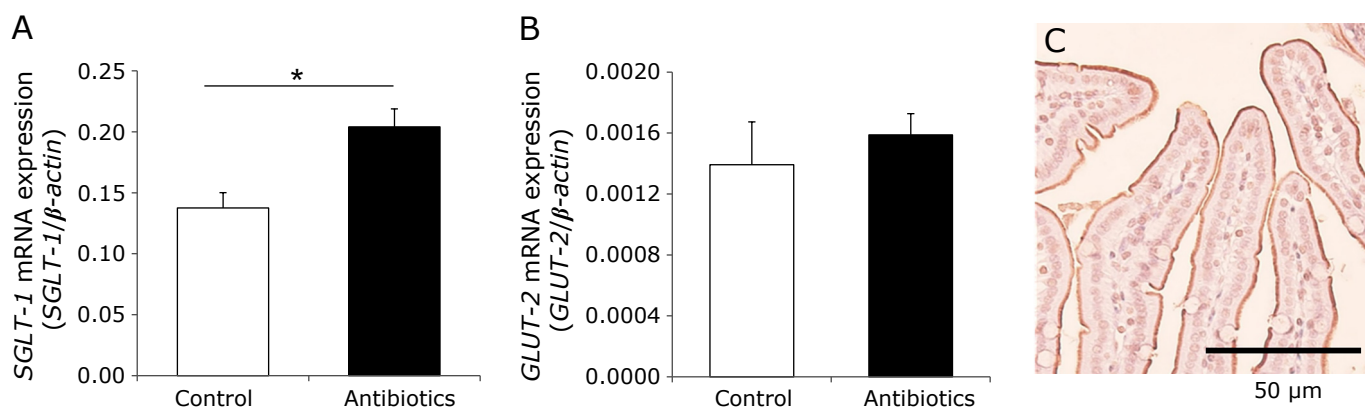


Fig. 2. *SglT1* and *Glut2* mRNA expression levels. (A) *SglT1* mRNA levels were significantly higher in the antibiotic-treated group than in the control group. (B) However, no differences in *Glut2* mRNA levels were observed in both groups. (C) SGLT1 was expressed mainly in the apical pole of epithelial cells in mice treated with antibiotics. Values are expressed as mean with SE of six mice in each group. * $p < 0.05$.

contributing to explain these associations are assumed. Lipopolysaccharide (LPS) from gut microbiota can induce a chronic subclinical inflammation, leading to insulin resistance, and short-chain fatty acids (SCFA) also have an essential role on the regulation of insulin sensitivity.^(27,28) Other mechanisms including bile acids and branched-chain amino acid (BCAA),^(29,30) all of whose composition are changed by gut dysbiosis, were also proposed, however, few studies have focused on the intestinal glucose transporters in the relationship between the gut microbiota and glucose homeostasis. The present results show that long-term use of broad-spectrum antibiotics upregulates jejunal

SGLT1. Furthermore, the jejunal villar height and surface area and CDX1 expression levels in the antibiotic-treated mice were significantly higher than those in the control group. To our best knowledge, our study is the first to show that long-term use of broad-spectrum antibiotics potentially affects the expression of glucose transporters in the small intestine.

SGLT1 is upregulated by DM,⁽¹⁶⁾ tacrolimus,⁽³¹⁾ α -ketoglutarate,⁽³²⁾ and vitamin D and downregulated by a low-carbohydrate high-protein diet or duodenojejunal bypass.⁽³³⁻³⁵⁾ Numerous factors alter SGLT1 expression levels in the small intestine; however, the mechanisms regulating SGLT1 remain

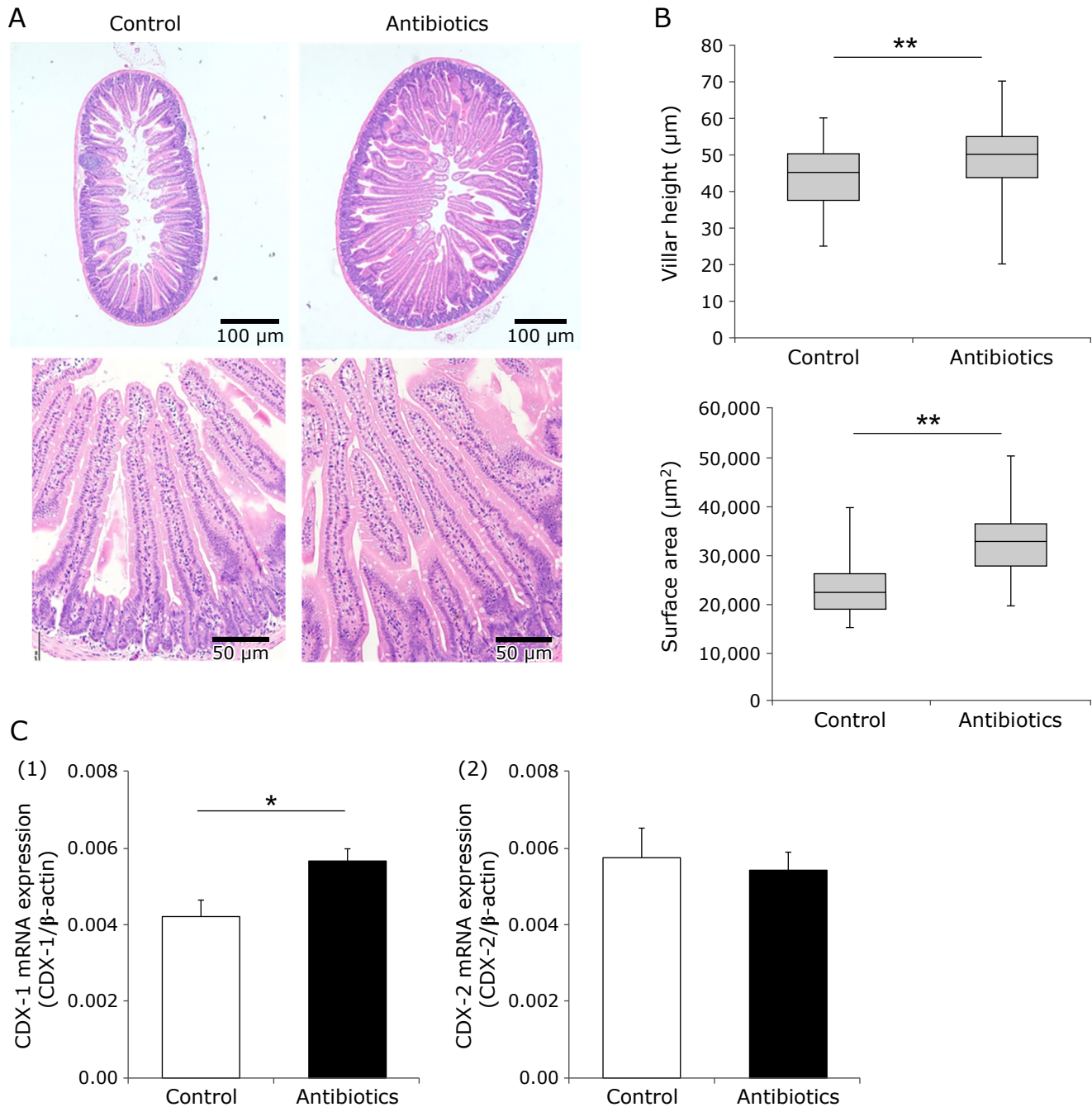


Fig. 3. Intestinal histology, and *Cdx1* and *Cdx2* mRNA expression levels. (A) Micrographs of representative intestinal sections from the two groups (hematoxylin and eosin staining, $\times 40$ magnification). (B) Villar height and villar surface area were significantly greater in the antibiotic-treated group than in the control group. (C) *Cdx1* mRNA levels were significantly higher in the antibiotic-treated group. (D) However, no difference in *Cdx2* mRNA levels were observed in both groups. Values are expressed as median with 25th and 75th percentiles (B) or mean with SE (C) of six mice in each group. * $p < 0.05$, ** $p < 0.001$.

unclear. This study shows that treatment with broad-spectrum antibiotics upregulates jejunal SGLT1, which is reportedly elevated in germ-free (GF) mice.⁽³⁶⁾ A major role of the gut microbiota is in energy extraction from undigested polysaccharides via the release of SCFAs. This energy extraction by the gut microbiota is lost in GF mice. Antibiotics-treatment also reduced the levels of microbial-derived SCFAs.⁽³⁷⁾ It is reasonable to assume that jejunal SGLT1 upregulation was induced to increase glucose absorption to compensate for reduced energy supply. Although the exact mechanisms under-

lying SGLT1 upregulation in this study remains unclear, it is possible that gut microbiota is directly or indirectly involved. Microbiome analysis revealed that the Firmicutes/Bacteroidetes ratio in antibiotic-treated mice was elevated, which is also observed in obese/insulin resistant individuals.⁽³⁸⁾ In line with our results, Adachi *et al.*⁽³⁹⁾ recently demonstrated that patients with type 2 DM had more *Lactobacillales*, belonging to the phylum Firmicutes, in their feces and less *Bacteroides* spp. compared to healthy individuals. Furthermore, it has been reported that SGLT1 expression in the small intestine is increased in obese

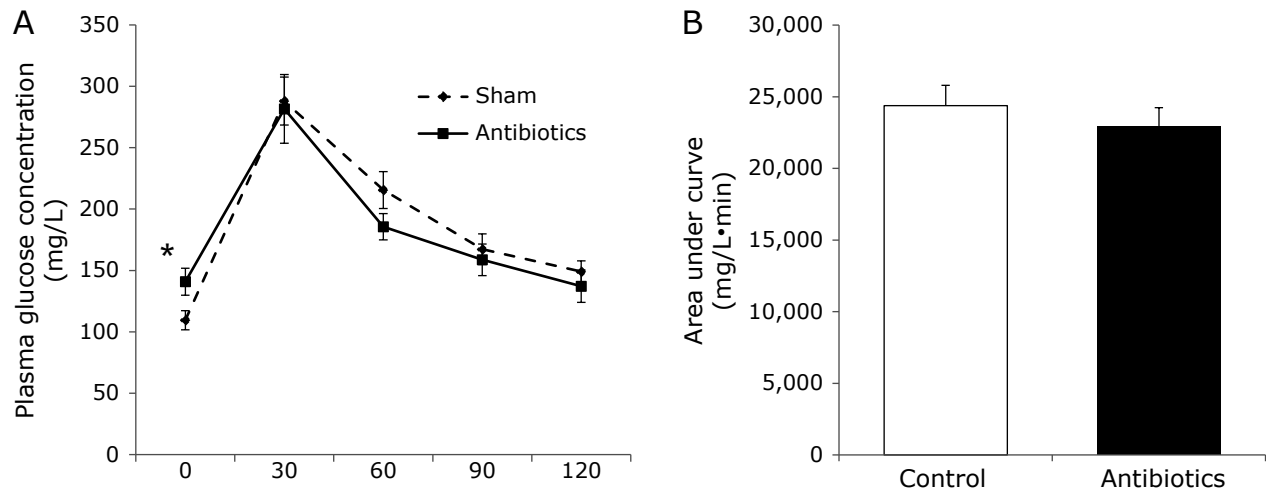


Fig. 4. Effect of antibiotics on plasma glucose levels. (A) Fasting glucose levels were significantly higher in the antibiotic-treated group than in the control group. However, no significant differences were observed at 30, 60, 90, and 120 min. (B) No significant differences were observed in the area under curve values for plasma glucose. Values are expressed as mean with SE of six mice in each group. * $p < 0.05$.

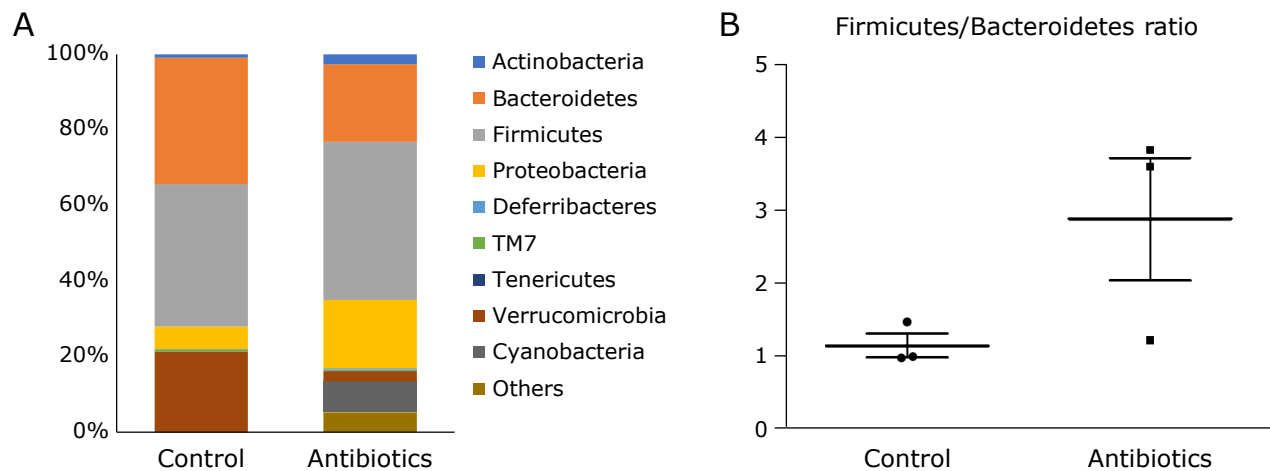


Fig. 5. Effect of antibiotics on gut microbiota. (A) The microbial composition at the phylum level. (B) Firmicutes/Bacteroidetes ratio. The Firmicutes/Bacteroidetes phylum ratio tended to be higher in the antibiotic-treated group than in the control group. Values are expressed as mean with SE of three mice in each group.

individuals.⁽⁴⁰⁾ Altered microbiota composition induced by broad-spectrum antibiotics treatment, including changes in the Firmicutes/Bacteroidetes ratio, may affect SGLT1 expression. The relationship between intestinal glucose transporter and gut microbiota and the mechanisms by which the gut microbiota can alter the intestinal SGLT1 expression need to be clarified by future studies.

Herein, fasting glucose levels were significantly higher in the antibiotic-treated group; however, the OGTT revealed no difference in glucose levels after a glucose load between the two groups. In the small intestine, SGLT1 is expressed in both enterocytes and enteroendocrine cells. Enterocyte SGLT1 is primarily responsible for glucose absorption, while glucose uptake by SGLT1 in enteroendocrine cells stimulates the release of incretins including glucagon-like peptide-1 (GLP-1).⁽⁴¹⁾ Glucose-induced incretin, GLP-1 and glucose-dependent insulinotropic peptide regulate blood glucose levels via several mechanisms, including amplification of glucose-induced insulin secretion. These findings suggest that SGLT1 upregulation potentially promotes incretin secretion after a glucose load. Differences in glucose levels in fasting and post-glucose loading

states between the two groups may be explained in part on the basis of the reactive secretion of incretin after a glucose load.

Furthermore, we found that jejunal villar height and surface area were significantly greater in the antibiotic-treated group than in the control group. Moreover, CDX1 expression levels were higher in the antibiotic-treated group than in the control group. Caudal-related homeobox genes *cdx1* and *cdx2* are intestinal transcription factors playing critical roles in intestinal cell proliferation and differentiation.⁽⁴²⁾ CDX expression in the small intestine is reportedly induced during starvation.^(43,44) Herein, fasting glucose levels and epididymal adipose weight were significantly lower in the antibiotics group than in the control group. Therefore, CDX1 expression and consequent jejunal epithelial hyperplasia might be induced in response to starvation-like conditions resulting from the reduction in the energy supply associated with antibiotic-induced dysbiosis. However, CDX1 expression and hyperplasia have been reported in the small intestine in a rat model of DM.⁽⁴²⁾ Since various factors are potentially involved in regulating CDX expression, and the microbiota may directly affect CDX expression, further studies are needed to elucidate the mechanism underlying antibiotic-

induced CDX expression.

In conclusion, our study showed that treatment with broad-spectrum antibiotics potentially upregulates SGT1 and CDX1 and induces hyperplasia in the jejunum, thereby increasing fasting blood glucose levels. Although the mechanism underlying the regulation of glucose homeostasis by the gut microbiota appears complex, our results indicate that SGLT1 is partially involved in glucose regulation via the gut microbiota. These findings further the current understanding of the effect of broad-spectrum antibiotics on the gut microbiota and glucose homeostasis.

Author Contributions

The conception and design of the work: TO and TI; acquisition of data: TO, TS, YE, SM, JY, YH, KM, and KO; analysis and interpretation of data: TO, TI, TD, TO, KI, KK, KU, TT, HK, YN, and YI; drafting the work: TO and TI. All authors approved the final version of this manuscript to be published.

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Abbreviations

AUC	area under the curve
DM	diabetes mellitus
GF	germ-free
GLP	glucagon-like peptide
GLUT	glucose transporter
OGTT	oral glucose tolerance test
SGLT	sodium-dependent glucose transporter

Conflict of Interest

No potential conflicts of interest were disclosed.

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