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Effects of Daikenchuto on Colonic Motility and Gut Microbiota in a Mouse Model of Hypoganglionosis

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Abstract

Congenital hypoganglionosis is a rare disorder characterized by a marked reduction in enteric ganglion cells, resulting in severe intestinal dysmotility. Daikenchuto (DKT), a traditional Japanese herbal medicine, is widely used for gastrointestinal disorders, but its efficacy in hypoganglionosis remains unclear. This study investigated whether DKT improves intestinal motility and modulates the gut microbiota in JF1/Ms mice, a murine model of congenital hypoganglionosis. Ex vivo colonic contractility was assessed using the Magnus method with stepwise DKT administration. In vivo, male and female mice were fed a diet containing 2.7% DKT for four weeks, followed by fecal microbiota analysis and bioconversion assays. DKT enhanced colonic contractility in JF1/Ms mice, although higher concentrations were required compared with wild-type B6 controls. Baseline microbiota analysis showed increased Bacteroidota, Campilobacterota, and Desulfobacteriota and decreased Firmicutes, resembling profiles reported in Hirschsprung's disease and intestinal obstruction. After DKT treatment, male mice showed a trend toward increased Bacteroides, whereas female mice exhibited increased Muribaculum. DKT also enhanced ginsenoside Rb1 metabolism in males. These findings suggest that DKT may exert therapeutic effects in congenital hypoganglionosis by improving intestinal motility and modulating the gut microbiome.

Introduction

Congenital hypoganglionosis is an infrequent enteric neuropathy characterized by a substantially reduced number of enteric ganglion cells within the myenteric plexus. This deficit in enteric innervation severely impairs intestinal peristalsis, clinically manifesting as chronic constipation, abdominal distension, and vomiting. Although categorized alongside other congenital enteric neuropathies, including Hirschsprung disease, congenital hypoganglionosis shows distinct histopathological features, including decreased ganglion cell density and hypoplastic enteric ganglia, which differentiate it from associated conditions [1, 2]. Symptomatic overlap with other disorders and low disease prevalence make diagnosis challenging. Although recent developments in immunohistochemistry using neuronal markers (e.g., HuC/D and CD56) have enhanced diagnostic accuracy [3, 4], therapeutic options remain restricted. Management is largely symptomatic; in severe cases, extended parenteral nutrition or small intestinal transplantation may be necessitated as definitive treatments [5, 6].

In Japan, Daikenchuto (DKT), a traditional Japanese herbal medicine (*Kampo*) comprising *Panax ginseng*, *Zingiber officinale*, *Zanthoxylum piperitum*, and maltose, is broadly utilized for numerous gastrointestinal disorders. Accumulating evidence indicates that DKT promotes intestinal peristalsis and increases intestinal blood flow by activating transient receptor potential channels on intestinal epithelial cells [7–10]. Moreover, DKT modulates gastrointestinal contractile activity via the interstitial cells of Cajal (ICCs), the pacemaker cells of the gut [11, 12]. Beyond its prokinetic effects, recent studies have suggested that DKT modulates the gut microbiota, increasing the proportion of short-chain fatty acid (SCFA)-producing bacteria, which contributes to enhanced epithelial barrier function and local inflammation regulation [13–18].

Considering these complex actions, DKT is frequently prescribed for a various symptoms, including constipation and abdominal bloating, and is administered perioperatively or in the management of inflammatory bowel diseases. DKT is considered a valuable therapeutic option in pediatric practice; notably, for congenital hypoganglionosis, where curative treatments are scarce, it serves as one of the few available interventions. However, the specific efficacy of DKT in the context of congenital hypoganglionosis and its impact on the disease-associated gut microbiota remain poorly understood despite its clinical application.

Therefore, this study aimed to investigate the effects of DKT on gastrointestinal motility and the intestinal environment using a murine model of congenital enteric ganglion cell reduction. We sought to elucidate the pharmacological basis for DKT's clinical utility in this rare neuropathy by evaluating physiological contractility and microbial shifts.

Results

Effects of DKT on Colonic Contractility in B6 and JF1/Ms Mice

Proximal colon (PC)

An organ-bath system with PC and distal colon (DC) segments excised from B6 and JF1/Ms mice was used for recording colonic contractions. Both B6 and JF1/Ms mice displayed a trend toward increased contraction frequency with higher DKT without maltose (DKT-e) concentrations. In B6 mice, contraction frequency was significantly elevated at 300 μg/mL compared with the pretreatment baseline ($p < 0.001$). The area under the contractile curve (AUC) normalized to tissue weight (wet weight) (AUC/W) peaked at 30 μg/mL, with a comparable pattern observed for the AUC per contraction. In JF1/Ms mice, the AUC/W exhibited an increasing trend at 100 μg/mL compared with baseline; however, it was not statistically significant ($p = 0.0697$) (Figs. 1a, 1c).

Distal colon(DC)

In the DC, both strains again demonstrated a trend toward increased contraction frequency at higher DKT-e concentrations. B6 mice showed a significant increase of contraction frequency at 300 μg/mL ($p = 0.0132$). Although the AUC/W peaked at 100 μg/mL, the increase was not statistically significant ($p = 0.0525$). The AUC per contraction was maximal at 30 μg/mL.

In JF1/Ms mice, contraction frequency was elevated at 100 and 300 μg/mL ($p = 0.0506$ and $p = 0.0463$, respectively), and the AUC/W peaked at 300 μg/mL ($p = 0.0525$) (Figs. 1b, 1d).

Comparison between Strains

Across all DKT-e concentrations, both the PC and DC exhibited a concentration-dependent decrease in contraction maxima and amplitude (difference between maxima and minima). In the PC, the AUC/W peaked at 30 and 100 µg/mL in B6 and JF1/Ms mice, respectively, compared with baseline. Notably, JF1/Ms mice exhibited consistently higher minimum contractile values in the PC than B6 mice at all concentrations, with the difference becoming more pronounced following DKT-e addition. Although the AUC/W did not differ at baseline, significant differences between strains emerged at 10 and 30 µg/mL ($p = 0.042$ and $p = 0.025$, respectively) (Figs. 1a–1d).

Differences in Gut Microbiota Composition between B6 and JF1/Ms Mice

Comparison of gut microbiota revealed substantial compositional differences between the two strains. JF1/Ms mice displayed higher abundances of the phylum Bacteroidota (including genera *Bacteroides* and *Alistipes*) and the phyla Campilobacterota and Desulfobacteriota, whereas the phyla Firmicutes and Actinobacteria were less abundant. Furthermore, the genus *Akkermansia* and members of the family Muribaculaceae were reduced in JF1/Ms mice (Suppl. Figure S1).

Alpha (α)-diversity assessed using the Shannon–Wiener index revealed no substantial difference in males, whereas female JF1/Ms mice demonstrated higher α-diversity than female B6 mice at 7 weeks (Suppl. Figure S3).

Beta diversity analysis by Principal Coordinate Analysis (PCoA) using Jaccard distance revealed a clear separation between the gut bacterial communities of B6 and JF1/Ms mice (Fig. 2).

Changes in Gut Microbiota following DKT Administration

At 11 weeks, the gut microbiota of JF1/Ms and B6 mice showed a significant difference, and additional changes were observed following DKT administration. In JF1/Ms mice, the relative abundance of the genus *Lachnoclostridium* (phylum Firmicutes) exhibited a decreasing trend at 11 weeks in the DKT-treated group compared with that in the untreated group (Suppl. Figures S2a–c).

In males, the genus *Tuzzerella* substantially increased over time in untreated JF1/Ms mice, whereas this increase was suppressed in the DKT-treated group. In females, the genus *NK4A214_group* sharply decreased in untreated JF1/Ms mice but remained at levels comparable to B6 in the DKT-treated group.

In both sexes, JF1/Ms mice demonstrated higher α-diversity than B6 mice. β-Diversity analysis confirmed clear differences between the B6 and JF1/Ms groups and between DKT-treated and untreated mice.

Linear discriminant analysis effect size (LEfSe) analysis identified group-specific microbial features. In males, the genus *Bacteroides* tended to increase with DKT treatment ($p = 0.076$), whereas females showed no significant changes. The genus *Muribaculum* revealed no group differences in males,

whereas in females, the DKT-treated group showed significantly higher abundance than the untreated group ($p = 0.035$) (Figs. 2a–f).

Enhanced Bioconversion of Ginsenoside Rb1 following DKT Administration

In the DKT-treated group, the residual fecal concentration of ginsenoside Rb1 at 11 weeks was lower than that at 7 weeks, indicating improved metabolism. This reduction was particularly significant in male JF1/Ms mice (Fig. 3b).

Correspondingly, total levels of Rb1 metabolites, including ginsenosides F2, Rd, and compound K (CK), were increased after 11 weeks in the DKT-treated group. This increase was also statistically significant in male JF1/Ms mice (Fig. 3c).

These findings indicate that the 4-week DKT administration improved the microbiota-dependent metabolic capacity of ginsenoside Rb1, particularly in male JF1/Ms mice.

Discussion

Our findings demonstrate the potential of DKT in enhancing intestinal motility even in JF1/Ms mice, a model of congenital hypoganglionosis [1, 2]. In organ-bath experiments, B6 and JF1/Ms colonic tissues demonstrated dose-dependent responses to DKT-e. However, JF1/Ms mice required significantly higher concentrations (100–300 μg/mL) to elicit responses comparable to those observed in B6 mice at lower concentrations (10–30 μg/mL), especially in the PC.

A major physiological insight from this study is that DKT does not simply act as a brute force stimulant. In B6 mice, although contraction frequency increased, the maximum amplitude tended to decrease, causing contractile waveform “remodeling” [12]. The synergistic actions of DKT’s constituent herbs my account for this reorganization. Specifically, hydroxy-α-sanshool from *Zanthoxylum* activates TRPV1 channels on extrinsic and intrinsic sensory nerve terminals, which can trigger excitatory neurotransmitter release and modulate spontaneous contractile activity [7, 8]. *Ginseng* saponins directly modulate the pacemaker activity of the ICC by increasing intracellular $Ca2+$ [11], whereas components from *ginger* and DKT facilitate acetylcholine release from cholinergic neurons, promoting a more sustained and rhythmic motor pattern [10, 12, 17]. Therefore, the observed extended waveforms in our *ex vivo* model potentially reflect a complex coordination of myogenic and neurogenic (cholinergic/sensory) pathways, independent of systemic vascular effects.

JF1/Ms and B6 mice displayed distinct differences in terms of contractile dynamics, most notably in the elevated minimum tension (basal tone) of the PC. This phenomenon can be interpreted through the lens of “denervation supersensitivity” or compensatory neuromuscular adaptation. In JF1/Ms mice, a

significant reduction in enteric ganglion cells and KIT-positive ICCs results in a failure in generating organized slow waves, which normally dictate a rhythmic 3–4-s interval in the PC [19, 20, 21].

Previous studies have reported that without sufficient pacemaker activity, the remaining enteric nerves in JF1/Ms mice undergo compensatory changes, likely encompassing extrinsic or intrinsic cholinergic fiber hypertrophy [22]. The high basal tone observed in our study may represent a state of sustained tonic contraction stemming from this uncoordinated neural input or a myogenic adaptation to chronic stasis. Although DKT successfully increased the contraction frequency in JF1/Ms mice, the narrow therapeutic window, as evidenced by the precipitous drop in amplitude at 300 μg/mL and complete cessation at 1,000 μg/mL, suggests that a “saturated” or “fatigued” neuromuscular state is easily reached in this model. Clinically, this finding implies that although DKT is effective for hypoganglionosis, the optimal dose must be cautiously titrated to avoid “over-excitation,” which may paradoxically inhibit effective propulsive motility by abolishing the necessary relaxant phase of the peristaltic cycle.

Microbiome analysis revealed that JF1/Ms mice harbor a distinct dysbiotic profile at baseline. The increase in phyla Bacteroidota, Campilogacteriota, and Desulfobacteriota alongside a decrease in phylum Firmicutes closely parallels the microbial signatures observed in Hirschsprung disease and chronic intestinal pseudoobstruction [23]. These changes may be secondary to impaired transit, which alters the luminal pH, nutrient availability, and oxygen tension.

The depletion of phylum Firmicutes (including some genera in order Clostridia and genus *Ruminococcus*) is particularly critical, as these taxa are the primary producers of SCFAs. SCFAs are not mere metabolic byproducts; rather, they are signaling molecules that stimulate enterochromaffin cells to release 5-HT, which in turn triggers the peristaltic reflex via 5-HT4 receptors on primary afferent neurons [24, 27]. Furthermore, SCFAs have been demonstrated to exert neuroprotective effects, promoting the survival and neurogenesis of HuC/D-positive neurons [25, 26]. The motility impairment in JF1/Ms mice potentially initiates a vicious cycle: reduced motility causes SCFA-producing bacteria loss, which reduces 5-HT signaling and neurotrophic support, further exacerbating the ENS dysfunction.

This study also revealed the sex-specific enrichment of the microbiota following the 4-week DKT treatment: genus *Bacteroides* in males and genus *Muribaculum* in females. This shift was correlated with the enhanced bioconversion of ginsenoside Rb1 into its active metabolites, particularly in males. These results suggest that DKT serves as a “prebiotic-like” modulator, selectively nurturing a microbial environment that optimizes its own pharmacological processing. Our findings align with recent studies demonstrating that DKT (TU-100) treatment significantly shapes the gut microbiota architecture and increases the production of the ginsenoside metabolite CK, a highly bioavailable and pharmacologically active derivative [28]. The gut microbiota plays a fundamental role in ginsenoside transformation, as parent saponins, including Rb1, are poorly absorbed in the upper gastrointestinal tract and require microbial enzymes, including β-glucosidase, for conversion into smaller absorbable metabolites, such as CK [29]. Previous studies on other Kampo medicines further support this concept; for instance, Bofutsushosan has been reported to significantly increase the abundance of *Akkermansia muciniphila* (*A.*

muciniphila), a beneficial bacterium associated with enhanced metabolic profiles in a mouse model of non-alcoholic fatty liver disease [30]. Similar to Bofutsushosan's effect on *A. muciniphila*, our findings indicate that DKT promotes the growth of specific taxa, genera *Bacteroides* and *Muribaculum*, which may play crucial roles in ginsenoside hydrolysis. This "microbiome–drug feedback loop" offers a novel scientific basis for the conventional Kampo concept that clinical efficacy frequently enhances and stabilizes with long-term sustained administration.

This study had several limitations. First, although our ex vivo experiments demonstrated the acute effects of DKT, the long-term structural recovery of the ENS and ICC network following the 4-week administration was not fully quantified. Second, although microbiome analysis suggested a "vicious cycle" involving SCFAs, we did not directly measure luminal SCFA concentrations; therefore, the functional association between microbial shifts and metabolite levels remained inferential. Lastly, although we identified sex-specific microbial enrichments, the underlying causes, including hormonal influences or sex-dependent bile acid metabolism, which drive these distinct profiles between males and females required further investigation.

In conclusion, our study suggests the potential of DKT in improving intestinal motility and supporting favorable microbiome adaptation, even in the setting of severe enteric neural abnormalities. However, the observed differences in strain sensitivity and the relatively narrow therapeutic window in the hypoganglionosis model highlight the potential significance of careful, individualized dose adjustments. Further research, particularly longitudinal studies integrating microbiome profiling with clinical symptoms, may contribute to the development of more personalized, microbiome-informed approaches for refractory gastrointestinal motility disorder management.

Methods

DKT and DKT-e

DKT-e was prepared by the aqueous extraction of an herbal mixture comprising dried ginger rhizome (Zingiber officinale Roscoe, Zingiberaceae), ginseng root (Panax ginseng C.A. Meyer, Araliaceae), and Japanese pepper pericarp (Zanthoxylum piperitum De Candolle, Rutaceae) in a ratio of 5:3:2 (w/w). The extract was manufactured by Tsumura & Co. (Tokyo, Japan). DKT was prepared by adding maltose at eight times the amount (by weight) relative to DKT-e.

Ethics declarations

All animal experiments were conducted following the relevant guidelines and regulations of each institution. Independent experimental studies were performed at Kyushu University and Tsumura & Co. The Institutional Animal Care and Use Committee of Kyushu University approved the animal procedures conducted at Kyushu University (Protocol Number: A24-324-2). Experiments performed at Tsumura & Co. complied with the Guidelines for the Care and Use of Laboratory Animals of Tsumura & Co. and were

approved by the Laboratory Animal Committee of Tsumura & Co. All procedures adhered to the guidelines for animal experimentation issued by the Ministry of Health, Labour and Welfare of Japan. All authors agreed that each is responsible for the accuracy and fairness of this study.

Animals

Animals used in colonic motility experiments

Male JF1/Ms mice (n = 3; National Institute of Genetics, Mishima, Japan) and male C57BL/6J (B6) mice (n = 3; KBT Oriental, Tosu, Japan) were purchased and used for intestinal motility experiments (total n = 6). All animals were 7–11 weeks old during the experiments. Mice were housed under controlled temperature conditions (21°C–23°C) with a 12-h light/dark cycle (lights on at 08:00 and off at 20:00); food and water were provided ad libitum (Oriental Yeast, Tokyo, Japan).

Animals used in gut microbiota and bioconversion experiments

Parent JF1/Ms mice (male and female) were obtained from the National Institute of Genetics (Shizuoka, Japan) and bred in-house. Six-week-old male and female C57BL/6J mice were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan), with 30 animals per sex used for these experiments (total n = 60). All animals were housed under controlled environmental conditions (23°C ± 2°C; relative humidity, 55% ± 10%; 12:12-h light/dark cycle; lights on 07:00–19:00) with ad libitum access to food and water.

At 7 weeks old, JF1 mice were randomly assigned to either a control group receiving a standard diet or a treatment group receiving a diet supplemented with 2.7% DKT (0.3% DKT extract without maltose plus 2.4% maltose). C57BL/6J mice were maintained on a standard diet only.

Tissue isolation and preparation for colonic motility

Three male mice each of the JF1/Ms and C57BL/6J (B6) strains were euthanized by cervical dislocation, followed by immediate laparotomy. The rectum was transected just above the peritoneal reflection, and the entire colon, including the terminal ileum, was excised by carefully detaching the mesentery as close to the intestinal wall as possible. The excised colon was immediately immersed in chilled oxygenated Krebs solution.

A small incision was created in the cecum, and a 20-gauge intravenous catheter sheath was gently inserted into the lumen. Care was exercised to avoid direct grasping of the intestinal wall and prevent excessive intraluminal pressure during handling. Following complete fecal content evacuation, the PC

was defined as the segment 1.5–3 cm distal to the cecal incision, and the DC was defined as the 1.5-cm segment proximal to the transected rectal stump. These defined segments were employed for subsequent functional analyses.

During laparotomy, all animals exhibited fecal retention. However, no substantial colonic dilation, tumor formation, or other gross anatomical abnormalities were observed.

The modified Krebs solution comprised 121.6-mM NaCl, 4.7-mM KCl, 2.5-mM $CaCl_2$, 1.2-mM $MgCl_2$, 1.2-mM $Na_2KH_2PO_4$, 15.4-mM $NaHCO_3$, and 11.5-mM D-glucose, gassed with 95% O_2 and 5% CO_2 (pH, 7.4 at 37°C).

Organ bath setup and contractile activity recording

Colon strips were vertically mounted in an automated four-chamber organ bath system (LE01046; ADInstruments, Bella Vista, NSW, Australia) equipped with MLT0420 isometric force transducers and a PowerLab data acquisition system. Each tissue segment was longitudinally attached to a tissue holder and set under an initial 1-g resting tension. The organ bath were filled with oxygenated (95% O2 and 5% CO2) Krebs solution maintained at 37°C continuously.

Tissues were allowed to equilibrate for at least 30 min, during which spontaneous colonic slow-wave activity was confirmed. Following the equilibration period, DKT-e was applied in a cumulative manner at 0-, 10-, 30-, 100-, 300-, and 1,000-μg/mL final concentrations. Changes in colonic contractile activity, including contraction frequency and strength, were continuously monitored.

Contraction strength was quantified on the basis of (1) maximum contraction amplitude, (2) minimum tension, and (3) the AUC calculated from the recorded tension waveform. All data were analyzed using LabChart software (ADInstruments).

Sample collection for Microbiome and Bioconversion analyses

Fecal samples utilized for microbiome and bioconversion analyses are summarized in Table 1. For bioconversion analysis, fecal samples were collected from male and female mice in three groups: C57BL/6J mice receiving a standard diet (B6 group), JF1 mice receiving a standard diet without DKT (JF1C group), and JF1 mice receiving a diet supplemented with 2.7% DKT (JF1D group). Ten samples per sex were obtained from each group. For microbiome analysis, 30 fecal samples per sex were collected from the same groups. Sampling was performed at two time points: before treatment (Week 7) and 4 weeks following DKT administration initiation (Week 11). This dataset facilitated a comparative evaluation of baseline microbial communities and treatment-induced alterations.

Table 1
Test Sample of Microbiome and Bioconversion analysis

Group	Sex	Number of Animals	
		MB	BC
B6 mice	Male	20	10
(non-treated group with Daikenchuto : B6)	Female	20	10
JF1 mice	Male	20	10
(non-treated group with Daikenchuto : JF1C)	Female	20	10
JF1 mice	Male	20	10
(2.7% Daikenchuto mixed feed traeated group : JF1D)	Female	20	10
Fecal Collection Period : Week7 and 11, MB : Microbiome analysis, BC : Bioconversion analysis			

Microbiome analysis

DNA was extracted from freeze-dried feces, and the V3–V4 region of the 16S rRNA gene was amplified by polymerase chain reaction using reagents from Takara Bio Inc. (Takara Bio Inc., Shiga, Japan). The amplified DNA (amplicons) was sequenced using paired-end sequencing on an Illumina MiSeq (Illumina, San Diego, CA, USA). The detailed procedure was previously reported [30].

To construct an amplicon sequence variant table clustered at 100% similarity, the obtained sequence data were processed using the QIIME2 package (version 2021.2.0) [31]. The taxonomic classification of the feature sequences was performed using the feature-classifier plugin against the reference sequence database (Silva 138 SSU).

The Shannon diversity index was used for assessing α-diversity (within-sample diversity), and Jaccard distance metrics was employed for evaluating beta diversity (between-sample diversity). Differentially abundant taxa among groups were identified using LEfSe [32]. Statistical comparisons of genus-level microbial abundances were performed using the Mann–Whitney U test with false discovery rate correction. All statistical analyses were performed using R software (The R Foundation for Statistical Computing, Vienna, Austria), Microsoft Excel, and QIIME2.

Bioconversion analysis

Materials

Ginsenoside Rb1 and diclofenac sodium were purchased from Fuji Film Wako Pure Chemical Corporation (Tokyo, Japan). Ginsenosides Rd, F2, and CK were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Asiaticoside was acquired from EXTRASYNTHESE (Lyon, France). Methanol (for LC/MS), acetonitrile (for LC/MS), formic acid, 1-butanol, and other general reagents were obtained from Fuji Film Wako Pure Chemical Corporation.

Bioconversion test of Ginsenoside Rb1

Metabolic capability was evaluated by measuring the production of ginsenosides Rd, F2, and CK, which are ginsenoside Rb1 metabolites. Metabolic reaction was performed by suspending fecal samples in 50-mmol/L phosphate buffer (pH, 7.0) at a 5-mg/mL concentration and subsequently incubating the suspension with ginsenoside Rb1 solution (final concentration, 5 μg/mL) at 37°C for 2 h.

Analysis of Ginsenoside RB1-Derived Components using LC/MS

Following the reaction, 300-μL 1-butanol and 20-μL of the internal standard solution (diclofenac, 250 ng/mL, 10 μL; asiaticoside, 1 μg/mL, 10 μL) were added and stirred for 1 min. Following the stirring, centrifugation was performed (10,000 × g, 4°C, 5 min) twice to collect 250 μL of the organic layer. The collected organic layers were combined (500 μL) and dried using a centrifugal evaporator (50°C; SpeedVac: SPD121P, OIL-FREE VACUMM PUMP: OFP400, Refrigerated Vapor Traps: RVT5105, Thermo Fisher Scientific K.K., Tokyo, Japan) and stored at − 20°C until analysis. Subsequently, 150 μL of 50% acetonitrile was added for redissolution. This solution was centrifugally filtered (5,000 × g, 4°C, 10 min) using Cosmospin Filter G (0.2 μm; NACALAI TESQUE, Inc., Kyoto, Japan), and the filtrate was used as the analytical sample. Furthermore, calibration samples were prepared by adding the compounds to a mouse blank fecal suspension at a 5-mg/mL concentration to achieve 0.004-, 0.01-, 0.02-, 0.04-, 0.1-, 0.2-, 0.5-, 1-, 2.5-, and 5-μg/mL final concentrations, followed by the same sample preparation procedure as the analytical samples. Ten microliters of both the calibration and analytical samples were analyzed using LC-MS/MS (Agilent 6460; Agilent Technologies, Santa Clara, CA, USA). The quantification ranges were 0.004–5, 0.004–5, 0.004–5, and 0.01–5 μg/mL for ginsenosides Rb1, Rd, F2, and CK, respectively. Detailed analysis conditions are provided in the Supplementary Information.

Calculation of concentration

The concentrations of each component were calculated using the peak area values of the internal standard (IS; diclofenac) with Mass Hunter Quantitative Analysis version 10.1 (Agilent Technologies, Santa Clara, CA, USA) based on the peak area ratios of each component. The calculated concentrations of each component (μg/mL) were converted to molar concentrations (μmol/L), and the ginsenoside Rb1

residual amount and ginsenosides Rd, F2, and CK production amounts were determined along with the total sum of the four components.

Statistical analysis

Statistical analyses were conducted using JMP 19 (SAS Institute, Cary, NC, USA) and GraphPad Prism 7.05 (GraphPad Software, San Diego, CA, USA).

For physiological motor activity (organ-bath experiments), data were expressed as the mean ± standard error of the mean from at least three independent determinations. Inter-group comparisons were conducted using two-tailed Student's t-test.

For gut microbiota and bioconversion analyses, temporal changes in ginsenoside Rb1 residual amounts and total metabolite production between 7 and 11 weeks were analyzed using the Wilcoxon matched-pairs signed-rank test. For comparisons of metabolite production across different experimental groups at 11 weeks, the Mann–Whitney U test with Bonferroni's correction for multiple comparisons was applied.

In all analyses, a p-value of < 0.05 was considered statistically significant.

Declarations

Competing Interests

S.I., Y.M., K.S., and M.N. are employees of Tsumura & Co.Tsumura & Co. provided partial funding for this study under a collaborative research agreement, and these authors contributed to a subset of the experiments and performed analyses related to their contributions.

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Author Contribution

R.I, J.K., and K.Y. led the ex vivo mouse model experiments for colonic motility experiments. K.S. and M.N. led the in vivo mouse model experiments for microbiome and bioconversion analyses. S.I. and M.N. performed microbiome analyses. Y.M. and M.N. performed bioconversion analyses. R.I. and N.K. wrote the manuscript. T.K., Y.T., A.F., J.M., J.M., K.N., T.M., and T.T. provided conceptual advice. All authors read and approved the final manuscript.

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Data Availability

All raw metagenomic sequencing data generated in this study have been deposited in the DDBJ Sequence Read Archive (DRA) under the BioProject accession number PRJDB42492. Additional processed data are available from the corresponding author upon reasonable request.

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Figures

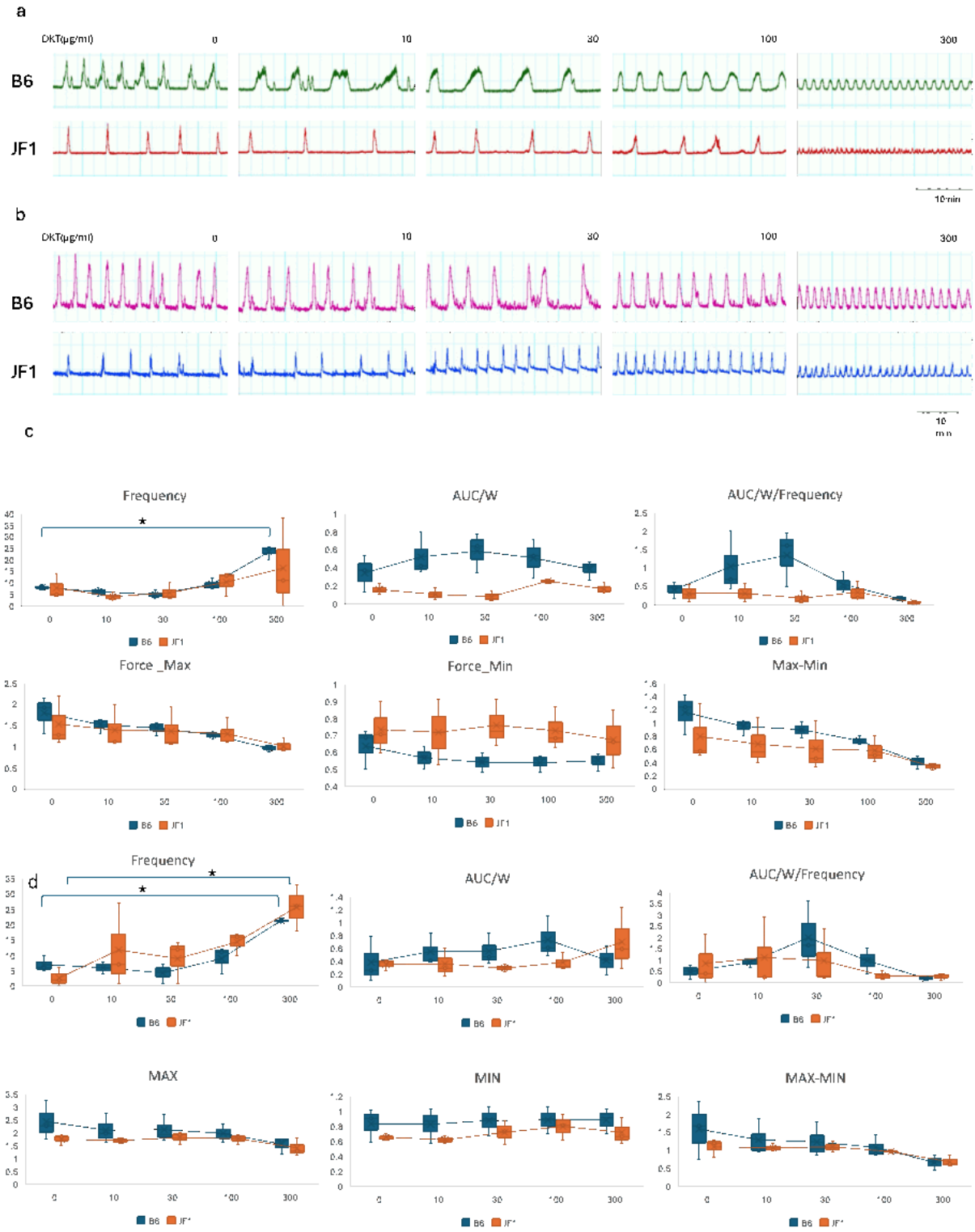


Figure 1

Concentration-dependent effects of Daikenchuto extract on colonic contractility in B6 and JF1/Ms mice

(a, b) Representative traces of spontaneous contractions in the proximal colon (PC) (a) and distal colon (DC) (b) from B6 and JF1/Ms mice during cumulative administration of Daikenchuto extract without

maltose (DKT-e; 0, 10, 30, 100, 300, and 1,000 μg/mL). DKT-e altered the contractile pattern in both strains, with B6 mice showing evident responses at lower concentrations and JF1/Ms mice requiring higher concentrations to exhibit comparable changes.

(c, d) Quantitative analysis of contractile parameters in the PC (c) and DC (d). In the PC, B6 mice showed a significant increase in contraction frequency at 300 μg/mL, whereas the AUC normalized to tissue wet weight (AUC/W) peaked at 30 μg/mL. In JF1/Ms mice, AUC/W tended to increase at 100 μg/mL, although this did not reach statistical significance. JF1/Ms mice also exhibited consistently higher minimum contractile values than B6 mice in the PC, and significant inter-strain differences in AUC/W were observed at 10 and 30 μg/mL.

In the DC, B6 mice showed a significant increase in contraction frequency at 300 μg/mL. In JF1/Ms mice, contraction frequency was elevated at 100 and 300 μg/mL ($p = 0.0506$ and $p = 0.0463$, respectively), and the AUC/W peaked at 300 μg/mL ($p = 0.0525$), suggesting that higher DKT-e concentrations were required to enhance distal colonic contractility in the hypoganglionosis model.

The following parameters were evaluated over a 30-min recording period: contraction frequency, AUC/W, AUC per contraction (AUC/W/frequency), maximal contractile value (MAX), minimal contractile value (MIN), and contractile amplitude (MAX–MIN). Data are expressed as mean ± standard error of the mean ($n = 3$ per group). Statistical comparisons were performed using two-tailed Student's t-test. *$p < 0.05$ versus baseline or between groups, as indicated.

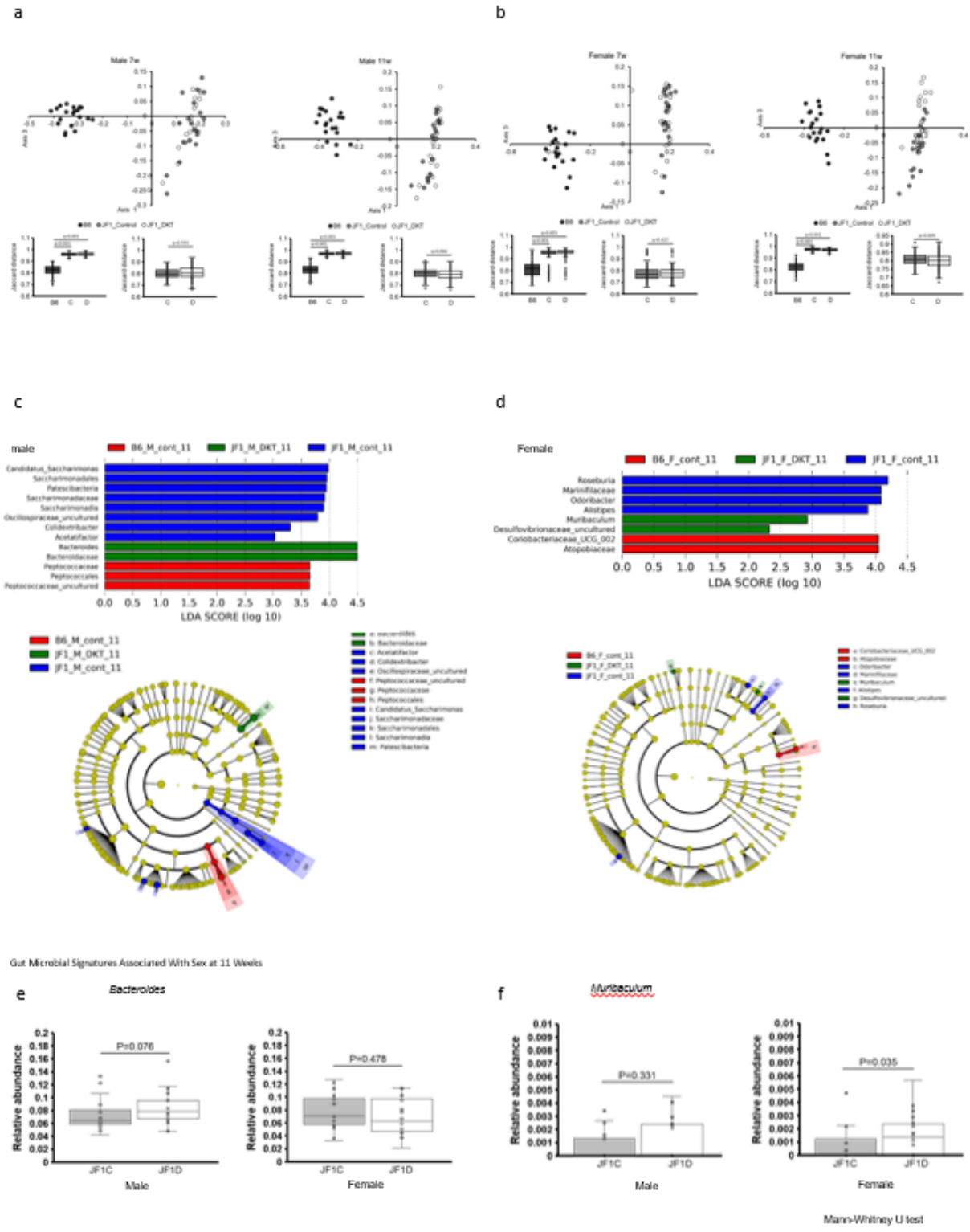


Figure 2

DKT-induced shifts in the gut microbiota of JF1/Ms mice at 11 weeks

(**a**, **b**) Principal coordinate analysis plots revealing the separation of gut microbial communities between the DKT-treated and untreated groups in (**a**) male and (**b**) female JF1/Ms mice. Each point represents an individual mouse (n = 10 per group). (**c**, **d**) Linear discriminant analysis effect size (LEfSe) identifying

significantly enriched or depleted microbial taxa in DKT-treated versus untreated groups in (**c**) males and (**d**) females. The threshold for the Linear Discriminant Analysis LDA score is set at >2.0. (e, f) Relative abundance of representative genera identified using LEfSe. (**e**) Genus *Bacteroides* enriched in male mice and (**f**) genus *Muribaculum* in female mice. Data are expressed as box plots. Statistical significance is determined using the Mann–Whitney U test. *p < 0.05 versus untreated control.

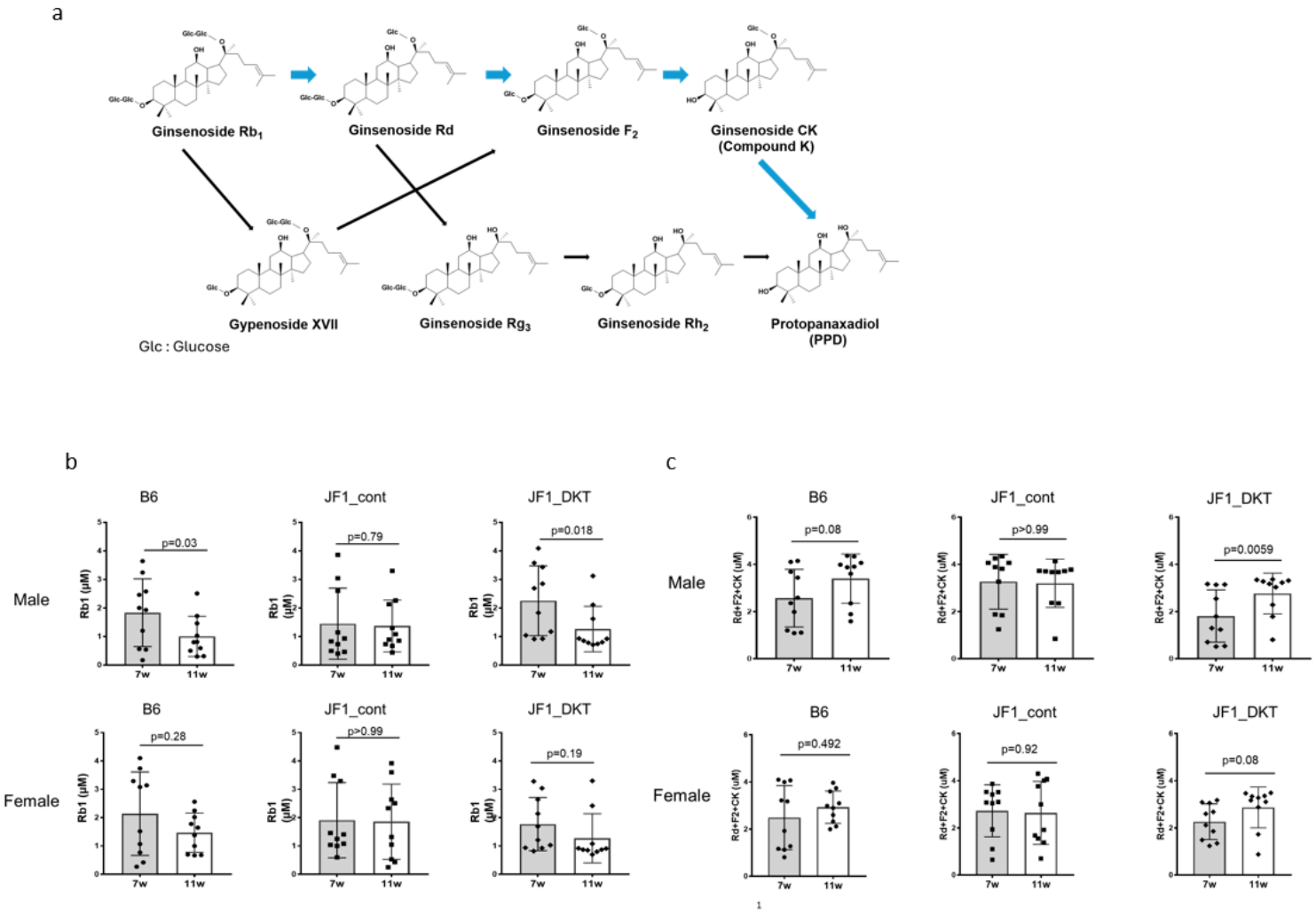


Figure 3

Enhanced ginsenoside Rb1 bioconversion following long-term DKT treatment

(**a**) Schematic representation of the metabolic pathway from ginsenoside Rb1 to its metabolites (ginsenoside Rd, F2, and Compound K) by the gut microbiota. (**b**) Ginsenoside Rb1 residual concentration in fecal samples at 7 weeks (baseline) and 11 weeks (after 4 weeks of DKT treatment) in JF1/Ms mice. (**c**) Total Rb1 metabolite concentrations in fecal samples. Results are stratified by sex, revealing more pronounced metabolic enhancement in males. Data are expressed as means ± standard deviations (n = 10 per group). Statistical significance is determined using the Wilcoxon matched pairs signed rank test. *P < 0.05 versus untreated control.

Supplementary Files

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