Original articles:

Predicting Inchinkoto efficacy, in patients with obstructive jaundice associated with malignant tumors, through pharmacomicrobiomics

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ABSTRACT

Inchinkoto (ICKT) is a popular choleretic and hepatoprotective herbal medicine that is widely used in Japan. Geniposide, a major ingredient of ICKT, is metabolized to genipin by gut microbiota, which exerts a choleretic effect. This study investigates the relationship between stool genipin-producing activity and diversity of the clinical effect of ICKT in patients with malignant obstructive jaundice. Fifty-two patients with malignant obstructive jaundice who underwent external biliary drainage were included. ICKT was administered as three packets per day (7.5 g/day) for three days and 2.5 g on the morning of the fourth day. Stool samples were collected before ICKT administration and bile flow was monitored on a daily basis. The microbiome, genipin-producing activity, and organic acids in stools were analyzed. The Shannon-Wiener (SW) index was calculated to evaluate gut microbiome diversity. The stool genipin-producing activity showed a significant positive correlation with the SW index. Stool genipin-producing activity positively correlated with the order *Clostridia* (obligate anaerobes), but negatively correlated with the order *Lactobacillales* (facultative anaerobes). Moreover, stool genipin-producing activity was positively correlated to the concentration valeric acid, but negatively correlated to the concentration of lactic acid and succinic acid. The change of bile flow at 2 and 3 days after ICKT administration showed significant positive correlation with genipin-producing activity (correlation coefficient, 0.40 and 0.29, respectively, $P<0.05$). An analysis of stool profile, including stool genipin-producing activity, may predict the efficacy of ICKT. Modification of the microbiome may be a target to enhance the therapeutic effect of ICKT.

Keywords

Obstructive jaundice, Kampo, Inchinkoto, genipin, microbiome, bio-conversion

Chemical compounds studied in this article

Inchinkoto (PubChem SID:51091259)

Geniposide (PubChem CID:107848)

Genipin (PubChem CID:442424)

Artemisiae Capillaris flos (PubChem SID: 135286652)

Gardeniae fructus (PubChem SID: 405231464)

Rhei rhizome (PubChem SID: 135325033)

Abbreviations

ICKT, inchinkoto; ENBD, endoscopic nasobiliary drainage; PTBD, percutaneous transhepatic biliary drainage; SW-index, Shannon-Wiener index; nMDS, non-metric multidimensional scaling; WBC, white blood cells

1. INTRODUCTION

Obstructive jaundice caused by a malignant tumor is a common symptom of the digestive system. Biliary obstruction may sometimes lead to hyperbilirubinemia, liver dysfunction, and endotoxemia, which are thought to be risk factors for in-hospital morbidity and mortality [1, 2]. Controlling serum total bilirubin levels before surgery is critical because hyperbilirubinemia substantially affects postoperative outcomes [3-7].

Inchinkoto (ICKT) (TJ-135, Tsumura & Co., Tokyo, Japan) is a popular choleretic and hepatoprotective herbal medicine, which is approved by the Japanese Ministry of Health and Welfare. ICKT includes Artemisiae Capillaris flos, Gardeniae fructus, and Rhei rhizoma. A previous randomized controlled study demonstrated the hepatoprotective activity of ICKT in patients undergoing major hepatectomy for malignancy [8]. Many other clinical and basic studies have reported a hepatoprotective effect following ICKT administration [9-19]. The mechanism underlying this activity of ICKT includes an upregulation of bilirubin transporter in the hepatocytes [18-20], an anti-inflammatory effect, and an anti-oxidative effect by inducing antioxidant enzymes [13]. Nonetheless, there is considerable individual diversity in the efficacy of ICKT for patients with obstructive jaundice associated with malignant tumors [8, 21]. Recently, we discovered a biomarker for predicting responders to ICKT using blood metabolites and found a relationship between the profile of gut microbiota and blood levels of genipin, one of the main active ingredients of ICKT [11, 21]. Geniposide, a major component of ICKT, has been shown to be metabolized to genipin by gut microbiota using an animal model [22]. It has been also shown that genipin exert choleretic effect through the upregulation of multidrug resistance-associated protein 2 (Mrp2) [20]. Therefore, it is hypothesized that the profile of gut microenvironment may have an impact on the metabolism of geniposide to genipin and affect a choleretic effect of ICKT. However, the metabolism of geniposide to genipin in human gut microbiota has never been investigated.

Recent advances in sequencing technology have revealed the crucial relationship between gut microbiota and host health and disease, as well as the large individual diversity of gut microbiota [23-25]. We reasoned that the gut microbiota profile could be correlated with the pharmacological action of ICKT, which might explain the observed diversity of patient response to ICKT. In this study, we measured the genipin-producing activity of stools collected from patients with malignant obstructive jaundice before ICKT treatment and investigated the potential relationship between genipin-producing activity in individual stools and the therapeutic response to ICKT.

2. MATERIALS AND METHODS

2.1. Study design

This study used stool samples collected in our previously report [26]. The study protocol was reviewed and approved by the Nagoya University Clinical Research Review Board (approval number: 2018-0496) and was registered with the Japan Registry of Clinical Trials (jRCT) under the registry number jRCTs041180158.

The original study participant recruitment and trial design have been fully described in the previous report [26]. Of the 54 patients recruited for the investigation, two patients dropped out during the study period (one chose to discontinue with the study after recruitment and the other failed to collect stool samples) and were excluded from the analysis. In brief, 52 patients with obstructive jaundice who underwent external biliary drainage either by endoscopic nasobiliary drainage (ENBD) or percutaneous transhepatic biliary drainage (PTBD) participated in this study (Figure 1). Drained bile was collected and replaced either by intake or through the use of a nasogastric tube. ICKT administration was not started until the amount of drained bile had stabilized (i.e., more than three days after drainage).

ICKT was administered as three packets/day (7.5 g/day) for three days and one

115 packet (2.5 g) on the morning of the fourth day. Stool, serum, and bile samples were collected
116 before administration of ICKT, which is indicated as “Pre” in the report. Thereafter, serum
117 and bile samples were collected one hour after the first (indicated as “Day 1”) and last
118 administration (indicated as “Day 4”) (Figure 1). The Bristol stool scale was used to classify
119 the form of feces into seven categories [27] and was monitored on a daily basis. The
120 illustration of the Bristol stool scale was distributed, and each patient was responsible for the
121 evaluation of stool form. The daily amount of bile flow collected in the drainage bottle was
122 monitored from pre to Day 4. For bile samples, the concentrations of total bilirubin, direct
123 bilirubin, and bile acid were measured. Serum samples were analyzed for the levels of
124 aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, direct
125 bilirubin, C-reactive protein (CRP), γ-glutamyl transpeptidase (γ-GTP), albumin, and alkaline
126 phosphatase (ALP). The stool samples were stored at -80 °C until use.

127 ***2.2. Gut microbiome analysis***

128 Bacterial genomic DNA was purified using a standard procedure with slight modifications
129 [28]. In brief, stool samples were freeze dried and 10 to 30 mg placed in a Lysing matrix E
130 tube (MP Biomedicals, Santa Ana, CA, USA). Stool samples were homogenized with elution
131 buffer using a FastPrep-24 automated cell disruptor (MP Biomedicals) with a speed setting of
132 6 m/sec for 40 sec. This disruption procedure was repeated twice in all. DNA was
133 subsequently isolated using a phenol/chloroform/isoamyl alcohol extraction procedure. The
134 16S rRNA gene metagenome library for MiSeq (Illumina, Inc., San Diego, USA) was
135 constructed according to the manufacturer’s protocol. The final library was sequenced using a
136 MiSeq Reagent Kit v3 (Illumina, Inc.) on the Illumina MiSeq platform. The sequence data
137 was processed as follows using a 16S rRNA sequence analysis pipeline, QIIME 1.8.0 [29].
138 Initially, both sides of the sequences were joined and those with a phred quality score of less
139 than 20 discarded. Chimera elimination by U-search was performed and contaminated

sequences removed. Open reference operational taxonomic unit (OTU) picking was carried out against green gene 97 13_8 as a reference dataset. A summary of taxonomy for each stool sample was derived using the script 'summarize_taxonomy_through_plots.py' in QIIME 1.8.0.

2.3. Measurement of genipin-producing activity in stool samples

In this study, we used ICKT as a substrate to estimate stool genipin producing activity to simulate the condition of patients who is administered ICKT. Measurement of stool bio-conversion activity was performed based on a previous study with some modifications [30]. Stool samples were weighed and suspended at 100 mg/mL in 50 mM phosphate buffer saline (pH 7.0). ICKT extract was dissolved in distilled water and adjusted to 200 mg/mL (Figure 2). A 30 μL aliquot of stool suspension was mixed with 3 μL ICKT solution in 267 μL sodium phosphate buffer (pH 7.0) and incubated at 37°C for 30 min. Reactions were quenched and extracted by addition of butanol (3x extractions using 300 μL each). The butanol recovered from the three extracts was pooled and dried using a centrifugal evaporator. Dried components were resuspended in 300 μL methanol. To prepare an 11-fold dilution, 20 μL of methanol resuspension was collected and added to 200 μL of 50% methanol. Niflumic acid, which was used as an internal standard, was mixed into the 11-fold dilution, and then diluted further with 10% methanol at a 1:20 ratio (v:v) before loading onto the solid-phase extraction (SPE) device (Oasis HLB; Nihon Waters K.K., Tokyo, Japan). The eluted solution from the SPE column was concentrated using a centrifugal evaporator. The dried residue was then dissolved in 100 μL of the specific HPLC mobile phase, and a 10 μL aliquot was injected into the LC-MS/MS system. The LC-MS/MS system consisted of a TripleQuad6500 (AB SCIEX, Tokyo, Japan) equipped with an Agilent 1290 system (Agilent Technologies Inc., Santa Clara, CA, USA).

2.4. Measurement of organic acids in stool samples

Fecal metabolome analysis was performed using the SGI-M100 derivatization system and GC-MS/MS. 10-20 mg of lyophilized feces samples were added to 1.2 mL of 80% acetonitrile containing 5 μg/mL crotonic acid as an internal standard. Samples were homogenized using zirconia beads in an automill (Tokken, Chiba, Japan) and then subjected to centrifugation before collecting the clarified supernatant for metabolome analysis. The analysis of organic acids was performed using a previously described procedure [31] on an automated derivatization SGI-M100 system (AiSTI SCIENCE, Wakayama, Japan). In this system, extracted samples were loaded onto an ion-exchange cartridge SPE. Target metabolites were retained in the SPE and derivatization was performed by direct addition of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide. The derivatized sample was subjected to GC-MS/MS analysis on a GCMS-TQ8040 (Shimadzu, Kyoto, Japan) system using a fused silica capillary column (BPX5: 30 m × 0.25 μm; film thickness, 0.25 μm; SGE, Melbourne, Australia). The front inlet temperature was 200°C and the flow rate of helium gas through the column was 39.0 cm/sec. The column temperature was held at 60°C for 3 min and then raised by 10°C/min to 100°C, then at 20°C/min to 310°C before maintaining this temperature for a further 1.5 min. The transfer line and ion-source temperatures were 290°C and 260°C, respectively. Organic acids were identified by comparing their peaks with authentic standards.

The peak intensity of each quantified ion was calculated and normalized to that of crotonic acid, which was used as an internal standard with a known weight of stool sample. Further analysis was performed using normalized values.

2.5. Statistical analysis

The Shannon-Wiener (SW) index was calculated using the script 'alpha_diversity.py' in QIIME 1.8.0 to evaluate the microbiome diversity of each stool [32, 33]. Beta diversity analysis was performed by non-metric multidimensional scaling (nMDS) using Bray-Curtis dissimilarity "metaMDS" using "adonis" in package "vegan" [34] in R 3.5.2 (The R

Foundation Conference Committee). Univariate analysis between two groups was performed with the Mann-Whitney U test using R 3.5.2. Spearman's correlation coefficients and uncorrelated tests calculated using R 3.5.2. A p value of less than 0.05 was considered statistically significant. SparCC correlation analysis [35] was performed using MicrobiomeAnalyst [36]. A Lasso (Least absolute shrinkage and selection operator) logistic regression analysis was performed to select microbiome species involved in genipin production using R *glmnet* package (parameters; family = "binomial", nfolds = 10, alpha = 1, nlambda = 100).

3. RESULTS

3.1. Patient characteristics

The median age of the 52 patients was 68 years and 85% of the patients (44/52) were male (Table 1). Most of the patients had biliary tract carcinoma and most of the patients underwent ENBD.

3.2. Genipin-producing activity in stool samples and the characteristics between the top 10 and bottom 10 subjects

Genipin is metabolized from geniposide which is included in the Gardeniae fructus and Gardeniae fructus is used as food additive in Japan. Indeed, some stool sample had genipin at 0 min (median 0.654 [0.229-1.728] μg/mL), but the stool genipin concentration levels were significantly increased after 30 min reaction (paired T-test <0.001). The average (standard deviation) and median (interquartile range) genipin-producing activity in stool samples were 3.8 (± 6.0) μg/mL and 1.44 (0.68-1.99) μg/mL, respectively. A histogram of stool genipin-producing activity for the 52 samples is shown in Figure 3. The distribution was considered multimodal rather than unimodal. Two stools out of 52 subjects produced over 10 μg/mL genipin, while 8 stools out of 52 subjects did not produce detectable levels of genipin within

30 min. This finding suggests that some patients produce a relatively large amount of genipin, whereas others produce only a small amount of genipin in response to ICKT administration. To clarify the characteristics of extensive and poor genipin producers, we selected the top 10 and bottom 10 subjects according to their stool genipin-producing activities. The Bristol stool scale, diversity of microbiome, and bile flow were compared between these two subgroups (Figure 4). For stool samples collected before ICKT administration (Pre), the Bristol stool scale for the bottom 10 (6 [4.5–6]) was significantly higher compared with the top 10 (3 [3–4], Figure 4A). The SW index in the bottom 10 (2.49 [2.09–2.91]) was significantly lower compared with that for the top 10 (3.75 [3.52-3.86]) (Figure 4B). These findings suggested that loose stool characteristics and poor microbiome diversity may be related to poor stool genipin-producing activity. The change of bile flow in each subject was calculated based on their levels at Pre. The change of bile flow at two days after administration of ICKT (on Day 2) in the top 10 (-40 [-82.5-0] mL) was significantly higher compared to the bottom 10 (85 [27.5-165] mL) (Figure 4C).

3.3. Stool microbiome and the relationship with genipin-producing activity

The relative abundance of the stool microbiome in all subjects are shown in order of genipin-producing activity (Figure 5A, Supplementary Table 1). The profile of microbiota for the bottom 10 patients, whose stool genipin-producing activity was below the detection limit or extremely low, was dominated by genera *Enterococcus* (0.273 ± 0.277)*, Lactobacillus* (0.067 ± 0.111)*,* or *Streptococcus* (0.029 ± 0.052)*,* which are facultative anaerobes. In contrast, the microbiome profile for the top 10 patients were dominated by obligate anaerobes (Supplementary Figure 1). nMDS was performed to investigate the relationship between the profile of microbes and stool genipin-producing activity. The subjects were clustered by stool genipin-producing activity along the nMDS1 axis. No gender bias was found in any clusters. These findings suggested the microbiome profile might affect genipin-producing activity

(Figure 5B).

3.4. Correlation analysis of gut microbiome, stool organic acids, and clinical parameters with stool genipin-producing activity

Next, we investigated correlation analysis using all 52 samples. The characteristics of patients with a variety of stool genipin-producing activities were analyzed using Spearman's correlation coefficient. Figure 6A shows microbiome composition of stool samples that were significantly correlated with stool genipin-producing activity. Specifically, stool genipin-producing activity displayed a significant positive correlation with 36 genera, and a significant negative correlation with 10 genera (Figure 6A, Supplementary Figure 2). Of the 36 positively correlated genera, 21 genera belonged to the order *Clostridia,* e.g., genera *Ruminococcus* (0.013 ± 0.020)*, Oscillospira* (0.006 ± 0.005), unknown genus in family *Ruminococcaceae* (0.055 ± 0.051) (Spearman's correlation coefficient; 0.68 [P<0.001], 0.68 [P<0.001], and 0.58 [P<0.001], respectively). By contrast, 4 out of 10 negatively correlated genera belonged to the order *Lactobacillales* e.g., genus *Enterococcus* (0.062 ± 0.161, Spearman's correlation coefficient; 0.43 [P=0.002]). SW index showed significant positive correlation with stool genipin producing activity (0.43 [P=0.002]). Further analysis between microbes were performed using SparCC. Significant correlation was not shown between order *Clostridiales* and *Lactobacillales* (SparCC correlation coefficient, -0.09 [P=0.693]). The strict anaerobes in phylum *Firmicutes* including order *Clostridiales* was positively correlated with each other as well as the facultative anaerobe in phylum *Firmicutes* including order *Lactobacillales* (Supplementary Figure 3) while correlation between strict anaerobes and facultative anaerobes were not significant except relationship between genera *Enterococcus* and *Oscillospira* (SparCC correlation coefficient; -0.51 [P=0.010]). It is suggested that both orders correlate stool genipin producing activity independently. Especially, genera *Ruminococcus* and *Oscillospira* were selected as candidate estimate markers by Lasso logistic

regression analysis (see Materials and Methods, coefficient; 0.35, genus *Ruminococcus*, and 0.20, genus *Oscillospira*).

Moreover, stool genipin-producing activity was positively correlated with the concentration of stool valeric acid (Spearman's correlation coefficient; 0.40 [P=0.003]), and negatively correlated with the concentration of stool lactic acid and succinic acid (Spearman's correlation coefficient; -0.28 [P=0.047] and -0.32 [P=0.019], respectively) (Figure 6B, Supplementary Figure 4A-D). The change of bile flow at 2 and 3 days after administration of ICKT showed significant positive correlation with genipin-producing activity (Spearman's correlation coefficient; 0.40 [P=0.004] and 0.29 [P=0.038], respectively) (Figure 6C, Supplementary Figure 5A, B). White blood cell (WBC) count and Bristol stool scale before administration of ICKT was also correlated with the stool genipin-producing activity (Spearman's correlation coefficient; -0.31 [P=0.027] and -0.41 [P=0.003], respectively) (Figure 6C, Supplementary Figure 5C, D).

The correlation between SW index and each organic acid concentration was determined. Formic acid and lactic acid showed a significant negative correlation with SW index (Spearman's correlation coefficient; -0.30 [P=0.030] and -0.30 [P=0.030], respectively). Valeric acid, isovaleric acid, isobutyric acid, and butyric acid showed a trend of positive correlation (Spearman's correlation coefficient; 0.26 [P=0.068], 0.25 [P=0.071], 0.22 [P=0.110], and 0.17 [P=0.228], respectively), whereas succinic acid showed a trend of negative correlation with SW index (-0.19 [P=0.185]) (Supplementary Figure 6).

4. DISCUSSION

To the best of our knowledge, this is the first report to reveal the relationship between the gut microbial profile and activation of a Kampo medicine using samples collected from patients. In this study, we focused on the relationship between the pharmacodynamics of

ICKT and stool genipin-producing activity, one of the bioactive compounds of ICKT, to characterize the diversity of pharmacological potency of ICKT. Stool genipin-producing activity varied widely among patients but showed significant correlation with: (1) Bristol stool scale, (2) diversity of intestinal microbiome, (3) abundance of certain microbes especially those of the order *Clostridiales* and *Lactobacillales*, and (4) stool organic acids. The stool genipin-producing activity was also correlated to bile flow volume changes observed 2 to 3 days after administration of ICKT.

Previous basic studies had demonstrated that genipin elicits bile secreting activities [19, 20]. Moreover, genipin is produced from geniposide, a major ingredient of ICKT, by gut microbiota [22, 37]. Thus, we reasoned gut microbiota may directly affect the production of genipin in the gut to alter the pharmacological effects of ICKT. However, this hypothesis had never been experimentally investigated using human samples. Here, we analyzed stool genipin-producing activity and revealed significant individual diversity between patients with obstructive jaundice. The distribution pattern of microbes as clustered according to stool genipin-producing activity by beta diversity analysis using nMDS (Figure 5B) indicated a potential relationship between genipin-producing activity and the stool microbiome. To clarify the characteristics of this relationship, we compared the top 10 and the bottom 10 subjects according to the stool genipin-producing activities. The top 10 subgroup showed a higher increase in bile flow two days after ICKT administration compared with the bottom 10 subgroup. These findings were validated by correlation analysis using whole samples (Figure 6). The results indicated that stool genipin-producing activity can be a potential biomarker for predicting the pharmacological effect of ICKT.

The bottom 10 subgroup in genipin-producing activity had a higher Bristol stool scale and lower microbiome diversity indicating that these patients had a dysbiotic condition. Generally, a healthy intestinal environment shows a higher diversity of stool microbiota

occupied by mostly obligate anaerobes (> 99%) [38]. An unhealthy intestinal environment tends to show lower diversity of stool microbiota comprising more facultative anaerobes and aerobes [39-41]. In addition, many studies indicate a relationship between low diversity of the microbiome and diarrhea [39-41]. Moreover, as well as low diversity, the shape of the microbiome in the bottom 10 subgroup comprised a lower abundance of obligate anaerobes and was instead dominated by facultative anaerobes such as bacteria of the genera *Enterococcus* and *Lactobacillus* that are known to produce lactic acid. Furthermore, SW-index was significantly negatively correlated with stool lactic acid concentration (Supplementary Figure 6). In the large intestine, lactic acid is metabolized to bioactive short chain fatty acids, such as acetic acid and butyric acid, primarily by obligate anaerobes. This metabolic transformation is inhibited in an imbalanced microenvironment with a lower abundance of obligate anaerobes, resulting in an accumulation of lactic acid in the stool. The findings in this study indicate that a dysbiotic condition associated with loose stools, reduced microbiome diversity, lower abundance of obligate anaerobes, and a lactic acid-rich microenvironment, may inhibit the metabolism of geniposide to genipin.

In addition to the stool lactic acid, the stool succinic acid concentration showed a significant negative correlation with stool genipin-producing activity. By contrast, the concentration of stool valeric acid showed a significant positive correlation with stool genipin-producing activity (Figure 6B and Supplementary Figure 4). Succinic acid is one of the metabolic products of the TCA cycle, which is utilized by aerobic and facultative anaerobic bacteria as an energy generating system. Therefore, high concentrations of succinic acid in the stool may represent the condition with more aerobic and facultative anaerobes, which are generally considered as pathogenic bacteria. Furthermore, other studies suggest a relationship between stool succinic acid and intestinal inflammation [42]. In contrast, gut valeric acid promotes the regulatory activity of lymphocytes by inducing IL-10 [43, 44]. In

addition, administration of valeric acid or its esters to animal models results in an anti-colitis effect [45]. Taken together, these findings suggest the stool concentration of lactic acid, succinic acid, and valeric acid is a useful biomarker not only for the dysbiotic condition, but also for stool genipin-producing activity as well as for predicting the pharmacological action of ICKT.

The results in this study indicate that a modification of the intestinal microbiome can be a potential target to improve the pharmacological action of ICKT. Indeed, fecal microbiota transplantation has been shown to improve the condition of dysbiosis [46]. Many previous studies also indicated that some probiotics or prebiotics formulations offset dysbiosis and improve the intestinal microenvironment. Our previous studies indicated that the preoperative use of synbiotics (a combination of prebiotics and probiotics) significantly increased the number of obligate anaerobes and decreased the number of facultative anaerobes/aerobes in the stool [47, 48]. The concentrations of organic acids were also improved by an administration of synbiotics. Therefore, it is recommended to co-administer synbiotics with ICKT when patients present with a dysbiotic condition to improve the pharmacological effect of ICKT.

Measurement of the conversion of glycoside to aglycon in stool samples seems to be a straightforward approach for predicting the likely biological activity of Kampo medicines. Despite the simplicity of this approach, it is thought to be useful in understanding the multiple pharmacological mechanisms of Kampo, which consists of numerous bioactive ingredients. Indeed, this approach can be used for other types of traditional medicine including over a hundred different Kampo medicines, which are commonly prescribed in Japan under the coverage of public health insurance. Kampo medicines comprise a crude mixture of herbal extracts of which glycosides are an important component. However, the hydrophilic properties of these glycosides makes them difficult to absorb. The gut microbiota is thought to

play an important role in metabolizing glycosides to aglycons, which are easily absorbed from the intestine into the portal circulation. Because there are large individual diversities in gut microbiota, stool metabolic activity for glycosides may depend on the profile of the microbiome for each patient. The approach described in this study will facilitate the identification of biomarkers for predicting the pharmacological effect of not only Kampo medicines, but also other traditional herbal medicines. If the relationship between intestinal environment and pharmacological activity of Kampo is further clarified, it may be possible to control the pharmacological activity of Kampo through regulating the intestinal microenvironment. In this study, stool genipin producing activity was not measured in strict anerobic condition and may be different from real human gut condition. Since no carbohydrate was added into the reaction mixture, and the reaction time 30 min seems to be relatively short, we consider that the shape of microbiota may not change dramatically in the reaction period. However, to obtain more accurate stool activity, further study will be needed.

There are some limitations in this study. Firstly, the sample size is small, necessitating additional larger scale studies to verify the results. Secondly, the correlation between stool genipin-producing activity and serum genipin level was not confirmed. It remains unclear whether the genipin-producing activity in the stool is linearly correlated to the blood genipin level and to the choleretic effect of ICKT. Thus, further studies are required to clarify the correlation between the gut microbiome profile and the pharmacological effects of ICKT.

5. CONCLUSIONS

In this study, we identified a relationship between stool genipin-producing activity and choleretic activity in patients who were administered ICKT. The stool genipin-producing activity was correlated with stool profile. The analysis of stool profiles, including microbiome diversity and organic acid concentrations, may be used to predict the pharmacological action

of ICKT. Modification of the stool profile may be a therapeutic target to enhance the pharmacological action of ICKT.

Declaration of Competing Interest

Y.Y. and M.N. received funding for this research from Tsumura & Co. H.Y. and T.E. have no conflict of interests to declare. M.N., K.O., H.K., and K.T. are employees of Tsumura & Co.

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564
565

FIGURE LEGENDS

Figure 1. Study design.

Fifty two patients with obstructive jaundice who underwent external biliary drainage participated in this study. Drained bile was replaced either by intake or through a nasogastric tube. ICKT treatment was started after the drainage had stabilized. The bile and serum samples were collected before (Pre), and 3 hours after administration of ICKT on Day 1 and Day 4. The stool samples were collected before starting ICKT administration.

Figure 2. Assay for stool genipin-producing activity.

ICKT was added into 10% (w/v) stool suspension and incubated at 37°C for 30 min. The reaction was quenched by adding 3 volumes of butanol and the concentration of genipin in the reaction mixture was subsequently measured by LC/MS/MS.

Figure 3. The distribution of stool genipin-producing activity.

Stool genipin-producing activity was measured as described in the Materials and Methods section. The distribution pattern is shown as a histogram.

Figure 4. Subgroup analysis of patients with the top 10 and bottom 10 genipin-producing activities.

(A) Bristol stool scale, (B) Shannon-Wiener index (SW index), and (C) bile flow increase on Day 2 were compared between the top 10 and bottom 10 patients based on stool genipin-producing activity. *; $P<0.05$.

Figure 5. Stool microbiome profile and genipin-producing activity.

(A) Relative abundance of the microbiome at the genus level in stools from each patient listed

591 in order of stool genipin-producing activity. (B) Non-metric multidimensional scaling was
592 performed for the top 10 subgroup (T10), bottom 10 subgroup (B10), and between the top 10
593 and bottom 10 subgroup (M).
594
595 Figure 6. Correlation analysis for stool genipin-producing activity
596 Correlation analyses between stool genipin-producing activity and (A) relative abundance of
597 microbiome, (B) stool organic acids concentrations, and (C) clinical data were performed
598 using Spearman's correlation analysis. Only values that gave a significant correlation ($P<0.05$
599 by uncorrelated test) are shown. Red bar; positive correlation. Green bar; negative correlation.
600
601
602 Supplementary Figure 1.
603 Profile of the gut microbiome and genipin-producing activity
604 Each genus is classified by its oxygen requirement and shown as a bar chart. The bar chart is
605 listed in order of stool genipin-producing activity. Blue; obligative anaerobe, orange;
606 facultative anaerobe + aerobe, gray; unknown.
607
608 Supplementary Figure 2.
609 Scatter plot of representative microbes showing significant correlation with stool genipin-
610 producing activity.
611 The relationship between genipin-producing activity and relative abundance of genera
612 *Ruminococcus* (A), *Oscillospira* (B), unknown genus in family Ruminococcaceae (C),
613 *Enterococcus* (D) shown as scatter plots.
614
615 Supplementary Figure 3.

Network analysis of gut microbiome in patient with obstructive jaundice caused by a malignant tumor.

The relationship between gut microbiome was performed using SparCC. The genus with a correlation coefficient greater than absolute 0.5 was selected ($P<0.05$).

Green; Bottom 10 subgroup, Purple; Top 10 subgroup, Red; between bottom 10 to top 10 subgroup.

Supplementary Figure 4.

Correlation analysis between stool organic acids and genipin-producing activity.

(A) Spearman's correlation analysis between stool organic acids and genipin-producing activity. Red; positive correlation, green; negative correlation. *; $P<0.05$ by uncorrelated test. Scatter plot for the organic acids showing a significant correlation with genipin-producing activity. (B) vs. stool valeric acid, (C) vs. stool lactic acid, (D) vs. stool succinic acid.

Supplementary Figure 5.

Scatter plot for clinical data and stool genipin-producing activity.

The relationship between genipin-producing activity and bile flow changes on Day 2 (A), the bile flow changes on Day 3 (B), the Bristol stool scale (C), and white blood cell (WBC) count before ICKT administration (D) shown as scatter plots.

Supplementary Figure 6.

Spearman's correlation analysis between stool organic acids and SW index.

Red, positive correlation; green, negative correlation. *; $P<0.05$ by uncorrelated test.

Supplementary Table 1.

Relative abundance of gut microbiota in individual patients with obstructive jaundice caused by a malignant tumor.

Table 1 Patient characteristics (n=52)

Age, years	68 (39–82)	
Sex, male / female	44 / 8	
Diagnosis, n (%)		
Cholangiocarcinoma	45	(87)
Pancreatic carcinoma	4	(8)
Hepatocellular carcinoma	1	(2)
Benign disease	2	(4)
Method of biliary drainage, n (%)		
ENBD	50	(96)
PTBD	2	(4)

645

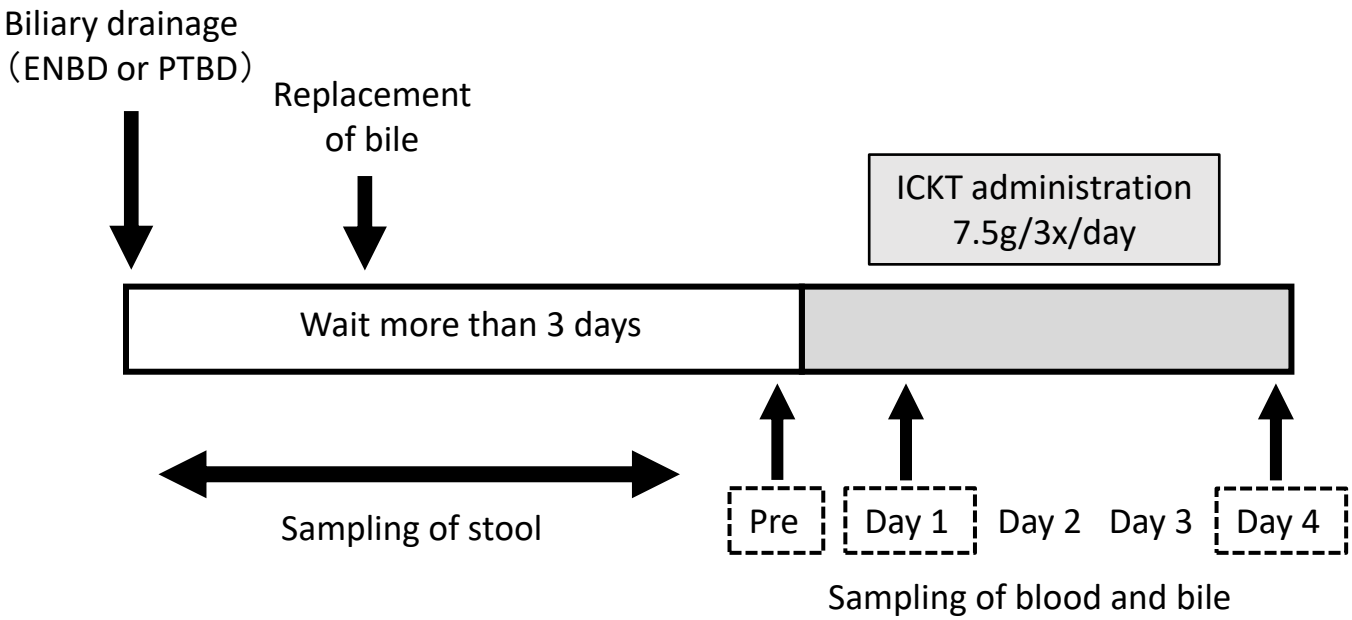

Biliary drainage
（ENBD or PTBD）
Replacement
of bile
ICKT administration
7.5g/3x/day
Wait more than 3 days
Sampling of stool
Pre
Day 1
Day 2
Day 3
Day 4
Sampling of blood and bile

Figure 1

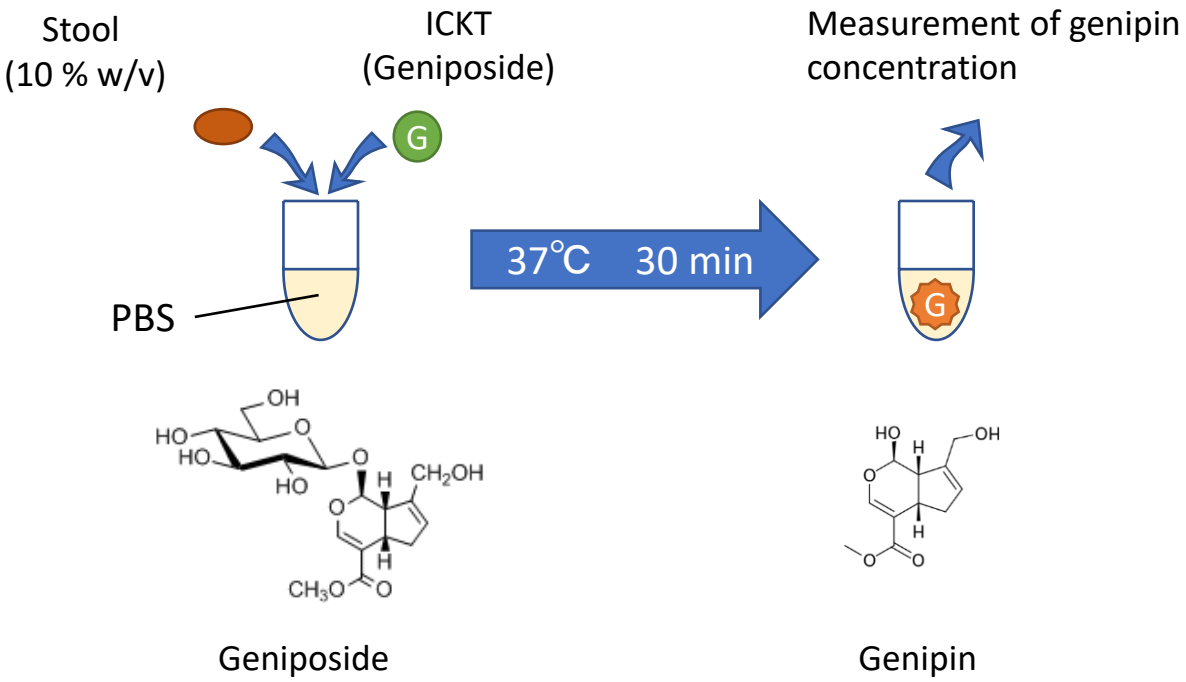

Stool
(10 % w/v)
ICKT
(Geniposide)
Measurement of genipin
concentration
G
PBS
37°C 30 min
G
OH
HO
HO
O
HO
O
H
CH2OH
O
H
CH3O
O
HO
H
OH
O
H
O
O
Geniposide
Genipin

Figure 2

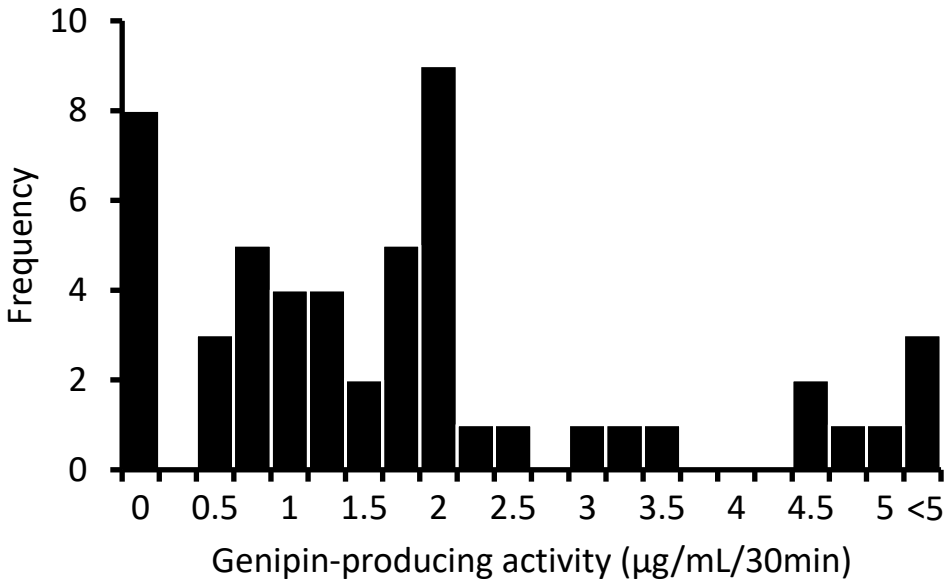

10
8
6
4
2
0
Frequency
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
<5
Genipin-producing activity (μg/mL/30min)

Figure 3

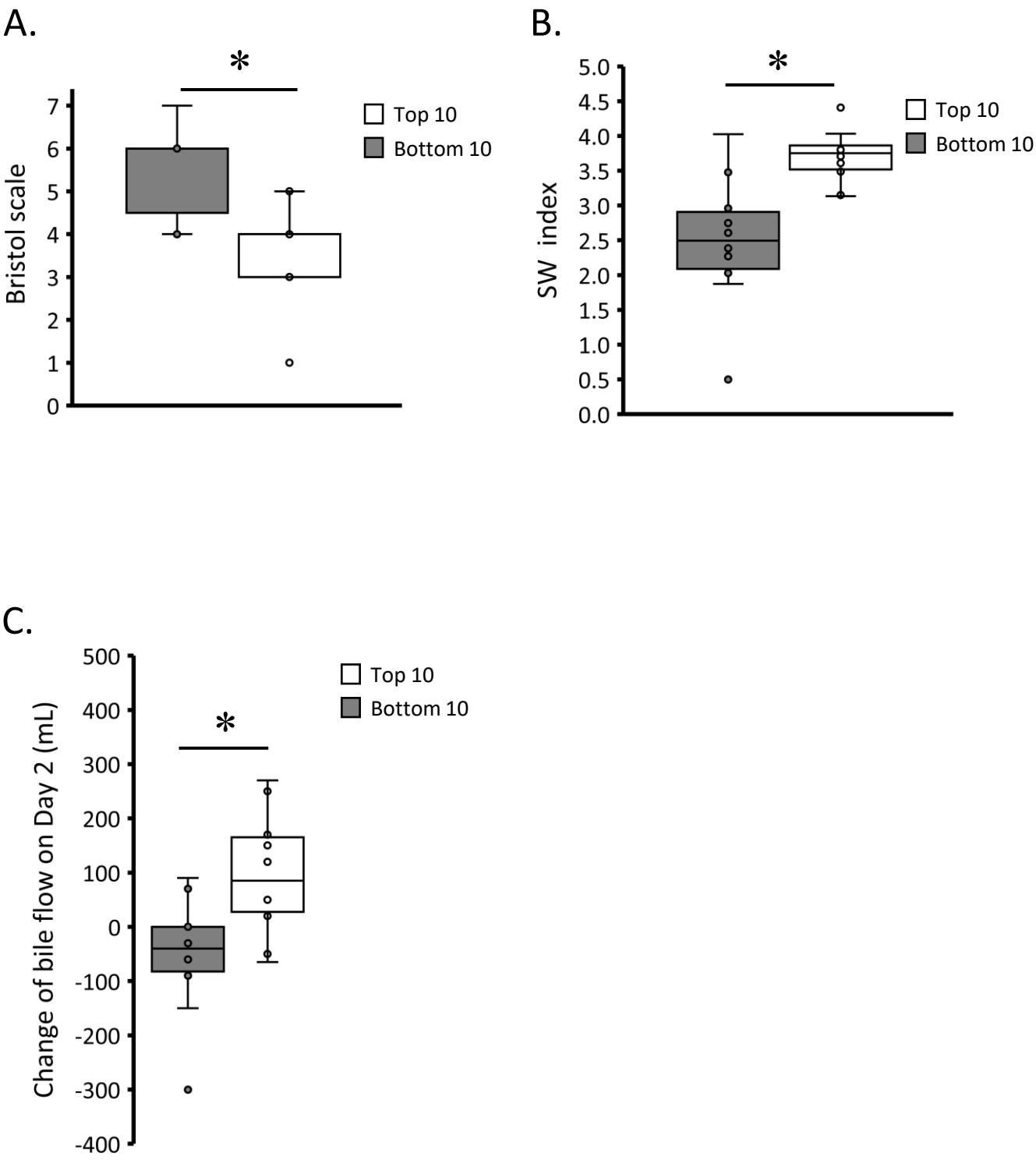
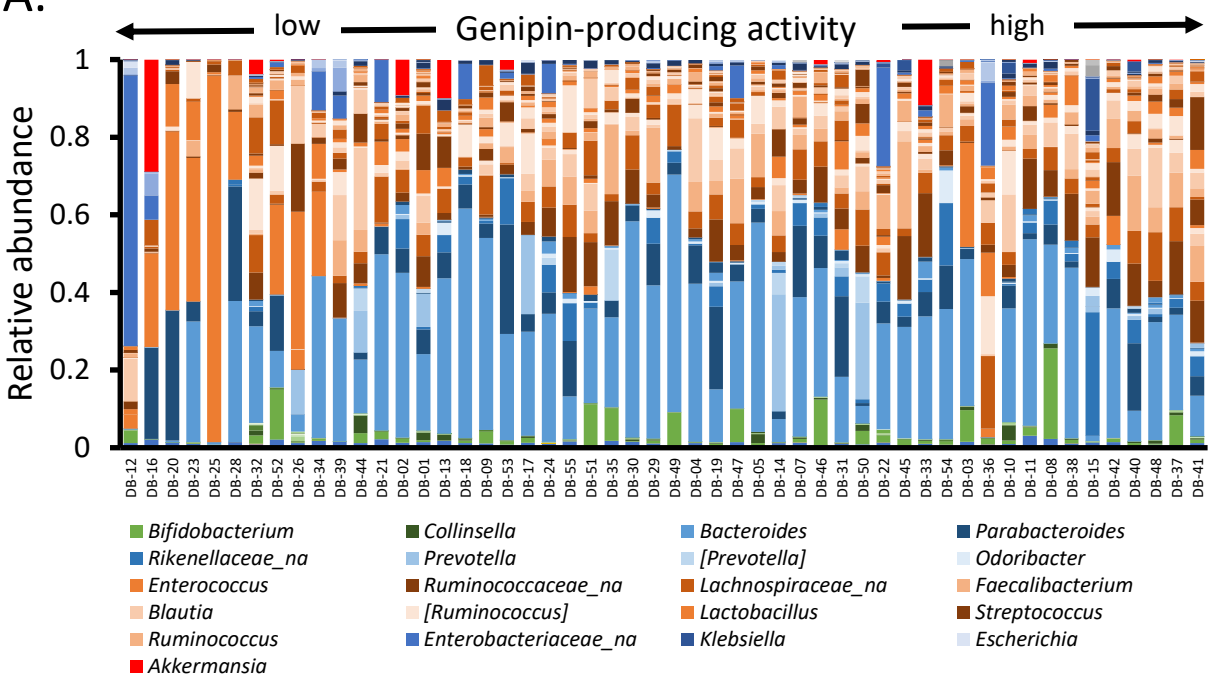

A.
*
Bristol scale
0
1
2
3
4
5
6
7
Top 10
Bottom 10
B.
*
SW index
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Top 10
Bottom 10
C.
*
Change of bile flow on Day 2 (mL)
500
400
300
200
100
0
-100
-200
-300
-400
Top 10
Bottom 10

Figure 4

A.


low
Genipin-producing activity
high
Relative abundance
1
0.8
0.6
0.4
0.2
0
DB-12 DB-16 DB-20 DB-23 DB-25 DB-28 DB-32 DB-52 DB-26 DB-34 DB-39 DB-44 DB-21 DB-02 DB-01 DB-13 DB-18 DB-09 DB-53 DB-17 DB-24 DB-55 DB-51 DB-35 DB-30 DB-29 DB-49 DB-04 DB-19 DB-47 DB-05 DB-14 DB-07 DB-46 DB-31 DB-50 DB-22 DB-45 DB-33 DB-54 DB-03 DB-36 DB-10 DB-11 DB-08 DB-38 DB-15 DB-42 DB-40 DB-48 DB-37 DB-41
Bifidobacterium
Collinsella
Bacteroides
Parabacteroides
Rikenellaceae_na
Prevotella
[Prevotella]
Odoribacter
Enterococcus
Ruminococcaceae_na
Lachnospiraceae_na
Faecalibacterium
Blautia
[Ruminococcus]
Lactobacillus
Streptococcus
Ruminococcus
Enterobacteriaceae_na
Klebsiella
Escherichia
Akkermansia

B.

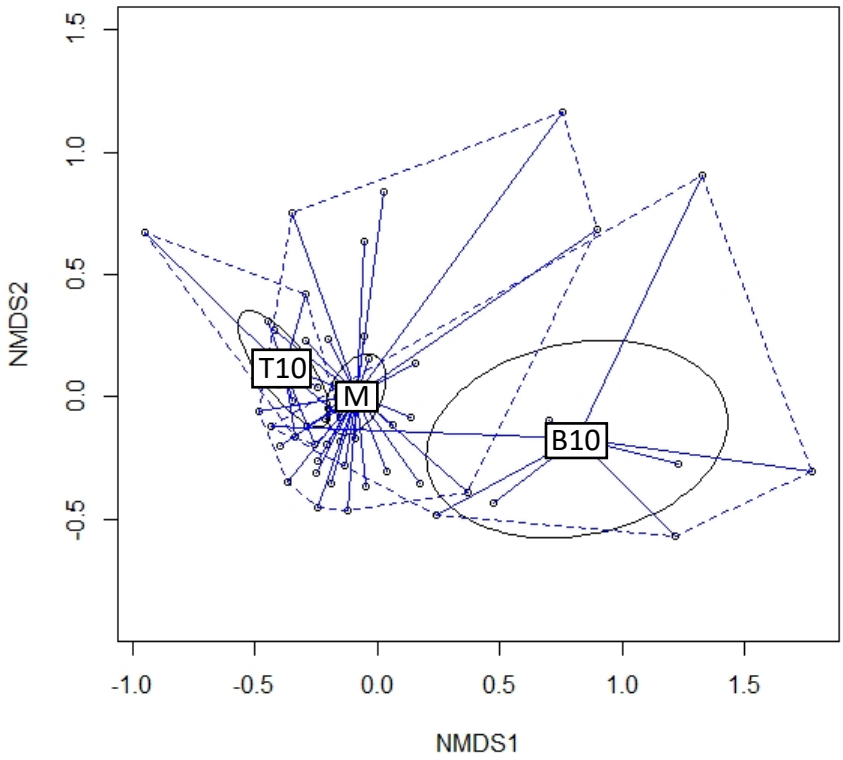

NMDS2
1.5
1.0
0.5
0.0
-0.5
T10
M
B10
-1.0
-0.5
0.0
0.5
1.0
1.5
NMDS1

Figure 5

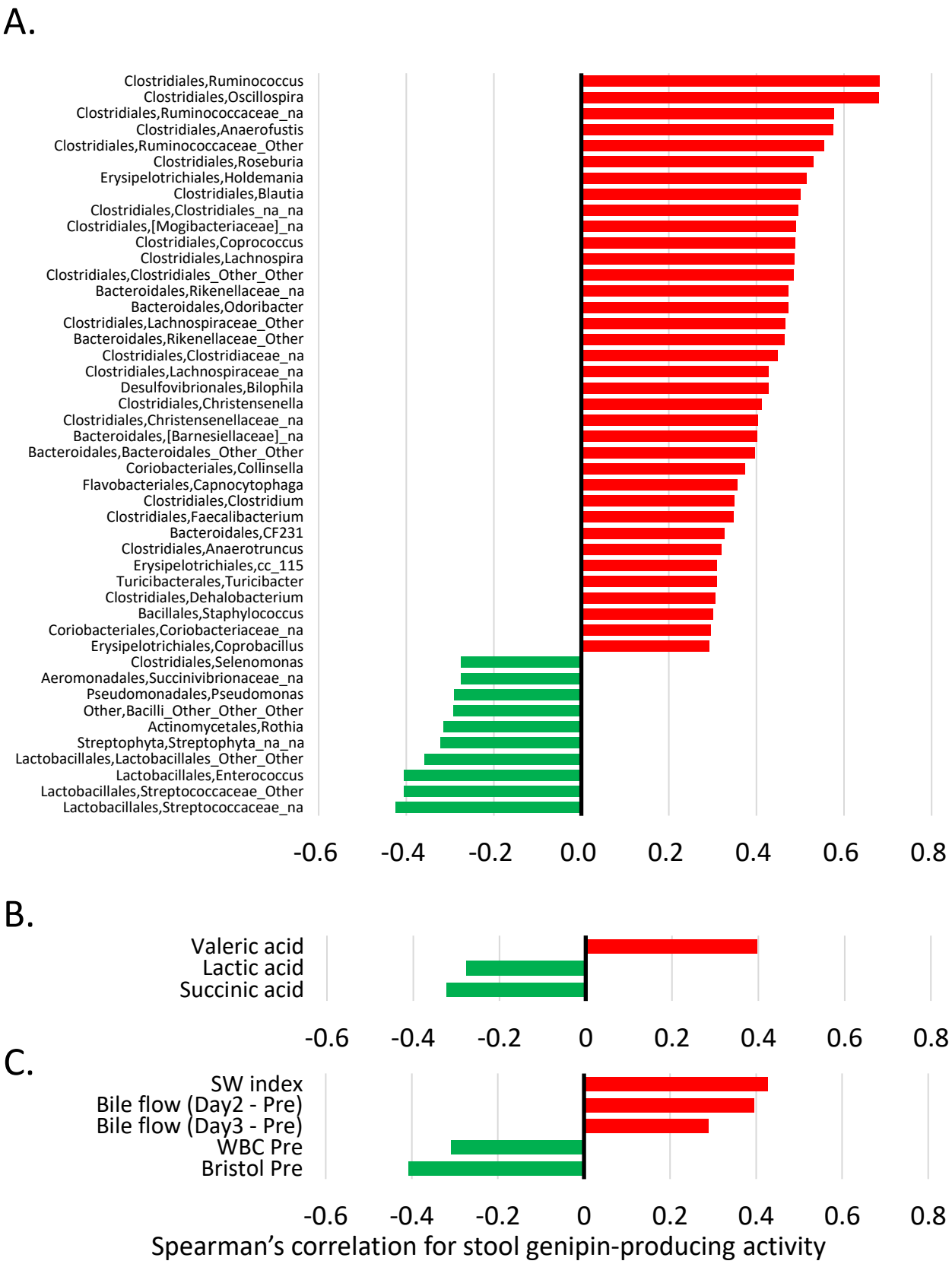
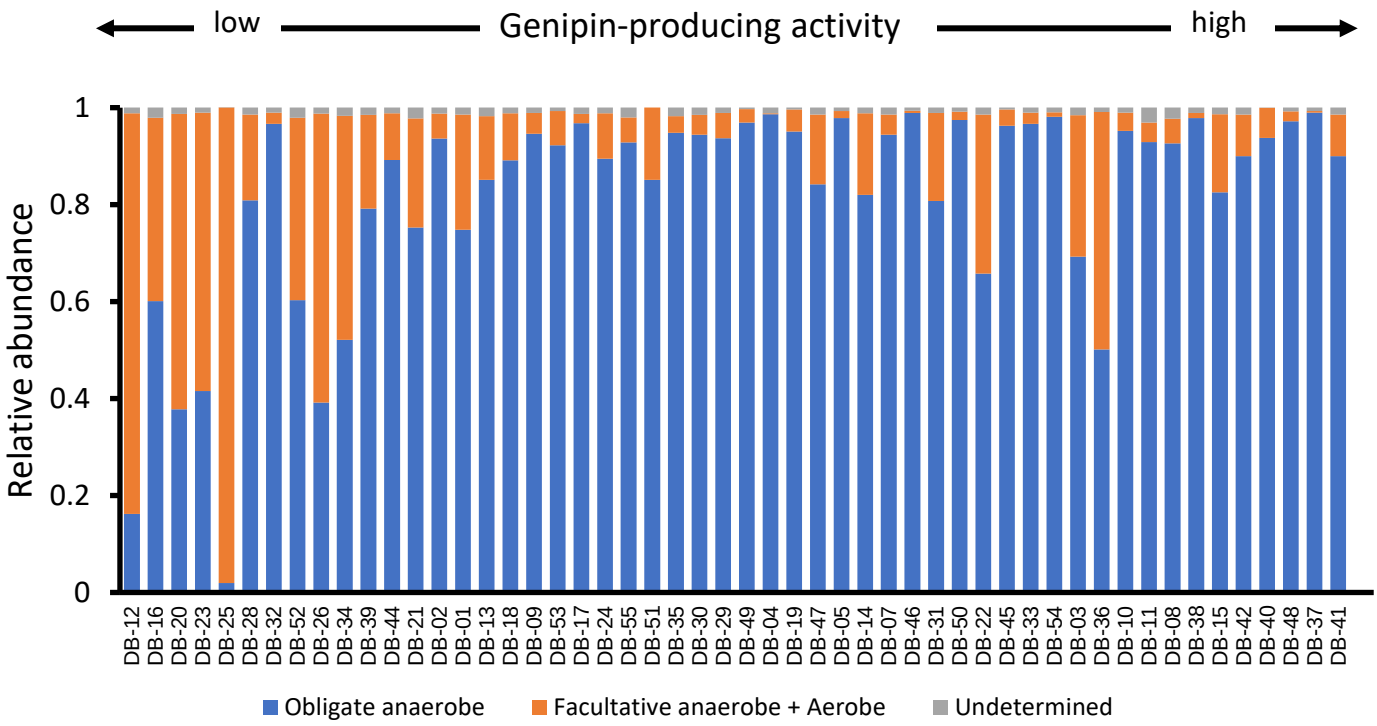
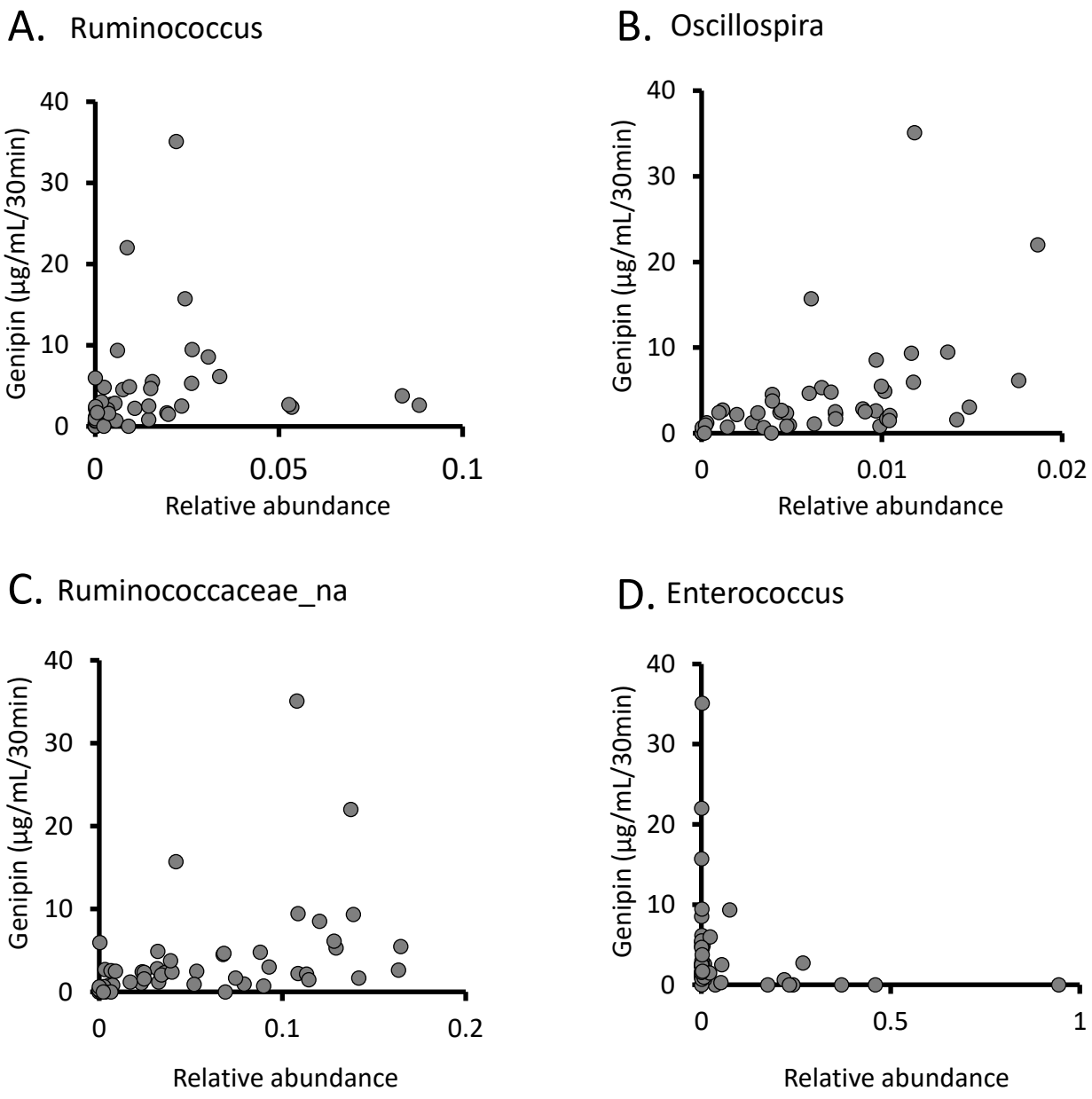


Figure 6. CにSW index を追記しました

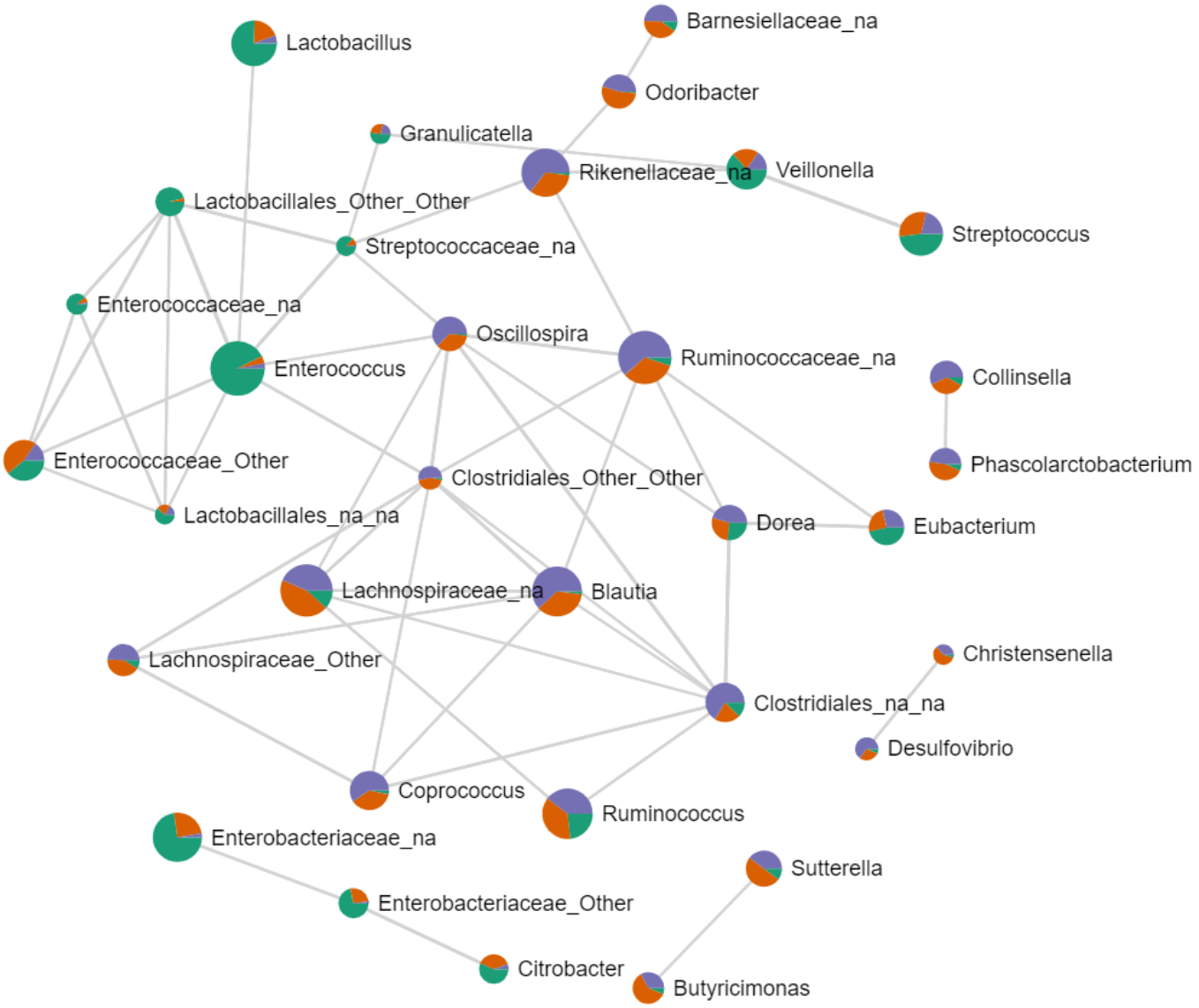
Figure 6



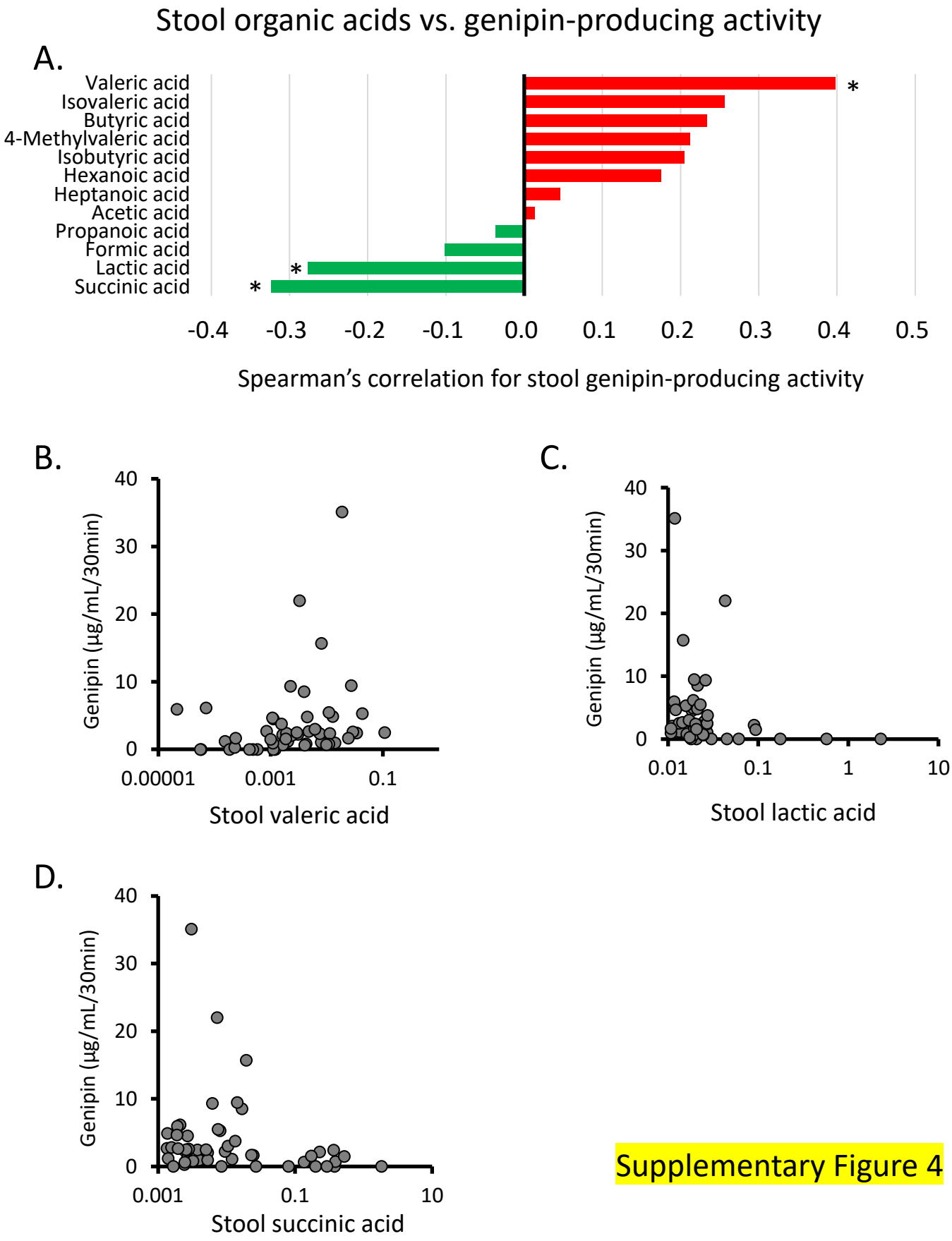
Supplementary Figure 1



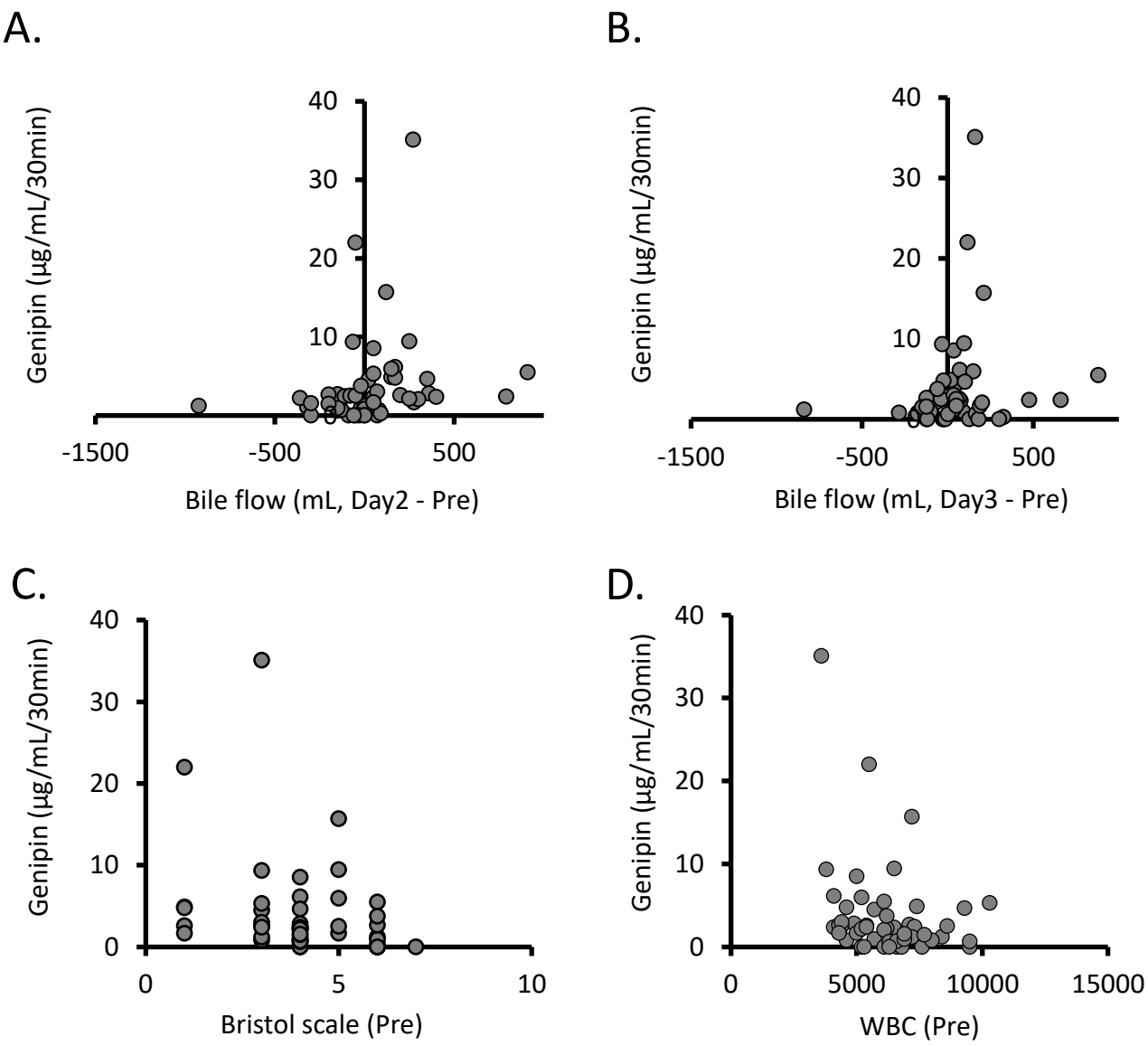
Supplementary Figure 2



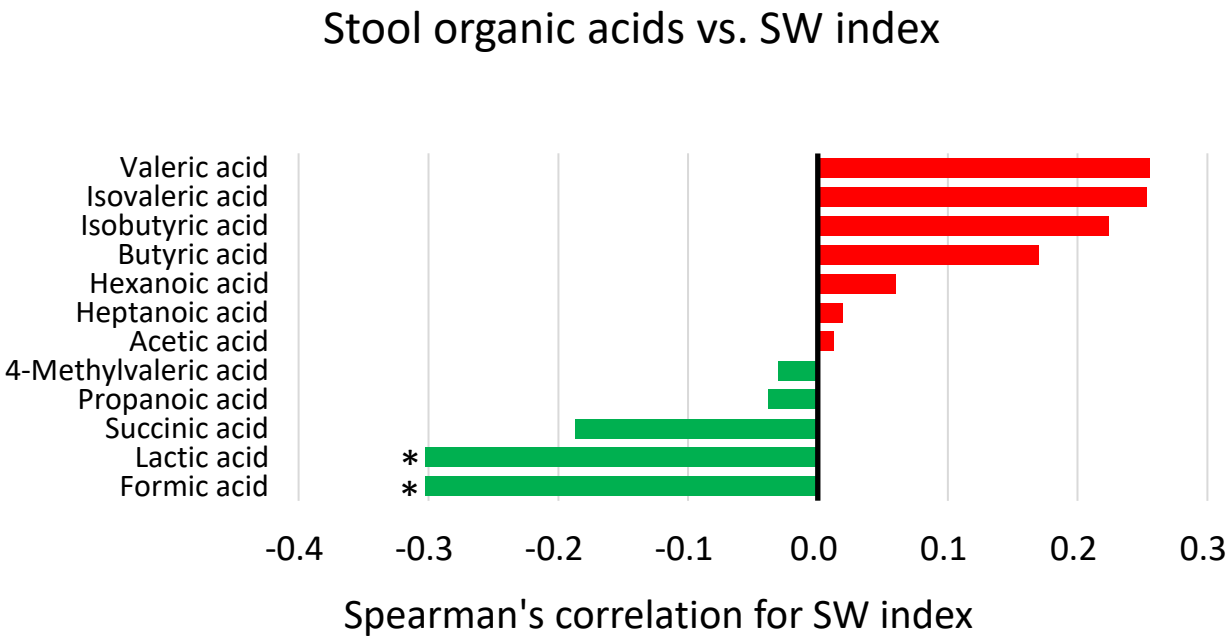
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6