

A newly developed solution for the preservation of short-chain fatty acids, bile acids, and microbiota in fecal specimens

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Recent studies have revealed that the gut microbiome affects various health conditions via its metabolites, including short-chain fatty acids (SCFAs) and bile acids (BAs). In the analysis of these, appropriate collection, handling, and storage of fecal specimens are required, and convenient specimen handling processes will facilitate their investigation. Here, we developed a novel preservation solution, "Metabolokeeper[®]", to stabilize fecal microbiota, organic acids including SCFAs, and BAs at room temperature. In the present study, we collected fecal samples from 20 healthy adult volunteers and stored them at room temperature with Metabolokeeper[®] and at -80°C without preservatives for up to four weeks to evaluate the usefulness of the novel preservative solution. We found that microbiome profiles and short chain fatty acid contents were stably maintained at room temperature with Metabolokeeper[®] for 28 days, while the bile acids were stably maintained for 7 days under the same conditions. We conclude that this convenient procedure to obtain a fecal sample for collecting the gut microbiome and gut metabolites can contribute to a better understanding of the health effects of fecal metabolites produced by the gut microbiome.

Key Words: preservation solution, intestinal microbiota, short-chain fatty acids, bile acids, fecal sampling

Accumulating research of the gut microbiome has revealed that the composition of human gut microbiota is linked to host health and disease.⁽¹⁻⁴⁾ Although the analysis of gut microbiota in feces has been focused on evaluating the health or pathological conditions of humans and animals, more emphasis has recently been placed on the analysis of metabolites produced by bacteria to evaluate the intestinal environment.⁽⁵⁾ In particular, short-chain fatty acids (SCFAs) and bile acids (BAs) are affected by alterations in the gut microbiota.^(6,7)

SCFAs are metabolites of dietary fiber and carbohydrates produced by the gut microbiota, also increased during the digestion of proteins and peptides.⁽⁸⁾ BAs are known to affect the gut microbiota owing to their high antibacterial activity. Primary BAs biosynthesized in the liver are converted to secondary BAs by intestinal bacteria.⁽⁹⁾ Therefore, to measure fecal metabolites, it is very important to immediately store collected fecal samples in frozen conditions to prevent metabolism by intestinal bacteria. The analytical values of metabolites from intestinal bacteria and the structure of gut microbiota depend on the length of time between the collection of the fecal sample and analysis, the temperature during storage, and the storage method, including

the preservation solution used.^(10,11) Therefore, it is important to develop a preservation solution that does not affect fecal metabolites or gut bacterial composition. In particular, a preservative solution that can be stored at room temperature for a long period could greatly contribute to research on the intestinal environment.

Regarding the storage method of fecal samples for the measurement of gut metabolites, some researchers have used the OMNIgene-GUT solution and RNAlater, storage solutions for bacterial flora analysis.^(12,13) In these studies, derivatized SCFAs were analyzed using GC-MS and LC-MS after storage at room temperature, and satisfactory results were not obtained. Here, we have already developed a preservation solution without bromothymol blue based on an existing guanidine thiocyanate solution for the preservation of fecal specimens,⁽¹⁴⁾ which consists mainly of a protein denaturant and can analyze SCFAs without derivatization. In the present study, we evaluated the effect of a newly developed preservative solution (Metabolokeeper[®]; TechnoSuruga Laboratory, Shizuoka, Japan) on the stabilization of fecal microbiota and organic acids, including SCFAs, and BAs, at room temperature.

Materials and Methods

Fecal sample collection. Feces were sampled from twenty healthy adult volunteers considered to be in good health (10 males: 39.7 ± 10.9 years old; 10 females: 35.2 ± 6.0 years old) at Kyoto Prefectural University. Fecal samples were collected without preservatives and stored at -80°C within one hour after defecation. After thawing to room temperature, 1.5 g of fecal material was weighed using a sterile swab and transferred into five tubes at room temperature, as shown in Fig. 1. One fecal sample out of a total of 20 samples was excluded due to deficiency of the necessary quantity; as a result, 19 samples were prepared for handling. One tube was supplied without intervention for fecal microbiota and organic acid analyses (D0), and one tube was frozen and stored at -80°C for 28 days (F28) for later analysis. Five ml of the newly developed preservative solution (Metabolokeeper[®]), containing guanidine thiocyanate and detergent, was added to the other three tubes. After vigorous mixing, the samples were stored at 30°C for 7 (D7), 14 (D14), or 28 (D28) days before analysis of SCFAs and BAs, and DNA extraction.

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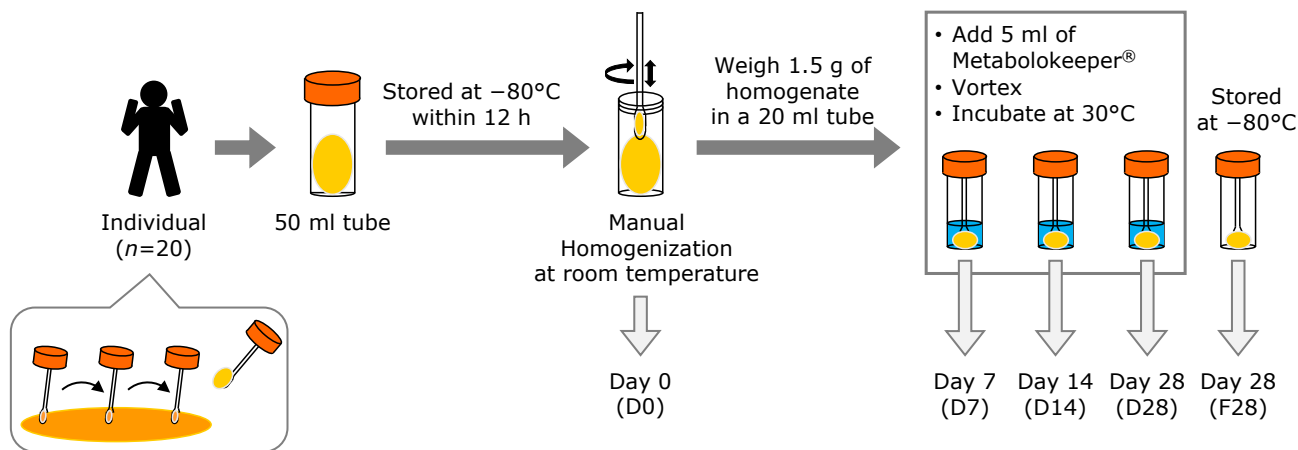


Fig. 1. Schematic of the study design including the workflow. Fecal samples were collected from 20 healthy adults and each fecal sample was divided into five aliquots. One aliquot was supplied as it was (D0), and another was frozen and stored at -80°C for 28 days (F28). Another three aliquots were stored in Metabolokeeper[®] at 30°C for 7 (D7), 14 (D14), and 28 (D28) days. The microbiota and metabolites in each specimen were analyzed.

This study conformed to the ethical code defined in the Declaration of Helsinki. The Ethics Committee of the Kyoto Prefectural University of Medicine approved the research protocol (approval number: ERB-C-1277-1), and all participants provided written informed consent before enrollment.

Analysis of SCFAs. Frozen fecal samples (F28) were thawed on ice. 100 mg of fecal samples (D0 and F28) or 300 μl of samples stored in Metabolokeeper[®] (D7, D14, and D28) were placed in 2.0 ml tubes with zirconia beads, and MilliQ water was added to the tubes up to 1 ml. The tubes were heated at 80°C for 15 min, vortexed at 5 m/s for 45 s using a FastPrep 24 5G (MP Biomedicals, Irvine, CA), and centrifuged at $18,400 \times g$ for 10 min. The supernatant was filtrated through a 0.2 μm filter (Ultrafree-MC; Merck Millipore, Billerica, MA). SCFAs (acetic acid, propionic acid, butyric acid, iso-butyric acid, succinic acid, lactic acid, formic acid, valeric acid, and iso-valeric acid) in feces were measured by high-performance liquid chromatography (Prominence; Shimadzu, Kyoto, Japan) using a post-column reaction with a detector (CDD-10A, Shimadzu), two tandem columns [Shim-pack SCR-102(H), 300 mm \times 8 mm ID; Shimadzu], and a guard column [Shim-pack SCR-102(H), 50 mm \times 6 mm ID; Shimadzu]. The LC system was used with a mobile phase (5 mM *p*-toluenesulfonic acid) and reaction solution (5 mM *p*-toluenesulfonic acid, 100 μM EDTA, and 20 mM Bis-Tris). The flow rate and oven temperature were 0.8 ml/min and 45°C , respectively. The detector cell temperature was maintained at 48°C . Measurements were performed using an absolute calibration curve (ranging from 5 to 1,000 mg/L). All analyses were performed using three aliquots from each tube. The data are presented as the average of triplicate analyses.

Analysis of BAs. BAs were extracted from fecal samples using a previously described method with minor modifications.⁽¹⁵⁾ 100 mg of fecal samples (D0 and F28) or 100 μl of samples stored in Metabolokeeper[®] (D7, D14, and D28) were placed in 2.0 ml-tubes containing zirconia beads and suspended in 900 μl of 50 mM cold sodium acetate buffer (pH 5.6)/ethanol mixture (1:3, v/v). The tubes were vortexed (5 m/s, 45 s) using FastPrep 24 5G (MP Biomedicals) and heated at 85°C for 30 min. After centrifugation ($18,400 \times g$, 10 min), the supernatant was diluted four times with MilliQ water and applied to a Bond Elute C18 cartridge (500 mg/6 ml, Agilent Technologies, Palo Alto CA). The cartridge was washed with 10% ethanol (5 ml), and BAs were eluted with ethanol (5 ml). The solvent was evaporated and the residue dissolved in 50% ethanol (1 ml). The

extracted solution was diluted with 50% ethanol, including an internal standard, and transferred to a vial after filtration using a 0.2 μm filter (Ultrafree-MC; Merck Millipore).

Quantification of bile acids was performed using a Waters Acquity UPLC system with an Acquity UPLC BEH C18 column (2.1 \times 150 mm, pore size 1.7 μm ; Waters Co., Milford, MA) coupled with a Waters Xevo G2-S QTOF mass spectrometer with an electrospray ionization probe. The injection volume was 4 μl . Mobile phase A was water and mobile phase B was acetonitrile, both containing 0.1% formic acid. The flow rate was 0.5 ml/min. The mobile phase was increased to 35% B from 0.5 to 1.0 min, increased to 40% B from 1.0 to 7.0 min, and increased to 50 from 7.0 to 10.0. The mobile phase was then changed to 95% B over 0.5 min and maintained for 1.5 min. The proportion was adjusted to the initial ratio of 30% B, and this was maintained for 4 min to equilibrate the column. The column and autosampler temperatures were maintained at 65°C and 10°C , respectively. The Waters Xevo G2-S QTOF was run in negative mode (scan 50–850 amu at a rate of 0.3 scans per second). The following instrument conditions were used: capillary, 0.5 kV; source temperature, 150°C ; sampling cone, 20 V; cone gas, 100 L/h; desolvation gas flow, 1,000 L/h at 450°C . Leucine enkephalin was used as the reference lock mass (m/z 554.2615) with a lock-mass spray to ensure mass accuracy and reproducibility. Data analysis was performed using TargetLynx software (Waters). All analyses were performed using three aliquots from each tube. The data are presented as the average of triplicate analyses.

DNA extraction. Fecal samples (100 mg; D0 and F28) or 100 μl of samples stored in Metabolokeeper[®] (D7, D14, and D28) were suspended in 900 μl GTC buffer [4 M guanidium thiocyanate, 100 mM Tris-HCl (pH 9.0), and 40 mM EDTA]. These samples were then beaten with zirconia beads using FastPrep 24 5G (MP Biomedicals). DNA was purified from bead-treated suspensions using GENE PREP STAR PI-480 (Kurabo Industries Ltd., Osaka, Japan). DNA concentrations were estimated by spectrophotometry using a NanoDrop 8000 (Thermo Fisher Scientific, Waltham, MA), and the final concentration of the DNA sample was adjusted to 10 ng/ μl .

Illumina library preparation, amplicon sequencing of 16S rRNA genes, and data analysis. Library preparation, including PCR amplification of the V3–V4 region of the 16S rRNA gene, was carried out as previously described.⁽¹⁶⁾ The forward and reverse primers also contained an 8-bp indexing sequence to allow multiplexing.⁽¹⁷⁾ Sequencing was conducted

using the Illumina MiSeq sequencing system and the MiSeq Reagent Kit v3 (600 cycle) chemistry (Illumina Inc., San Diego, CA). Paired-end sequencing reads were merged using the fastq-join program with default settings.⁽¹⁸⁾ The joined amplicon sequence reads were processed using the QIIME2 software.⁽¹⁹⁾ Quality filtering and deletion of chimeric sequences were performed, and representative sequences were created using DADA2 (Divisive Amplicon Denoising Algorithm 2) denoise-single plugin ver. 2017.6.0, with default settings.⁽²⁰⁾ The taxonomy of representative sequences was assigned using Greengenes database ver. 13.8 by training a naive Bayes classifier using the q2-feature-classifier plugin.⁽²¹⁾

Statistical analysis. The Shannon index for alpha diversity was calculated using the QIIME2 diversity alpha-rarefaction plugin. Bonferroni-corrected pairwise Wilcoxon tests were executed to compare the Shannon index among the three groups (D0, D28, and F28) using R.⁽²²⁾ For beta diversity, principal coordinate analysis (PCoA) was performed based on Bray–Curtis distances using the QIIME2 diversity core-metrics-phylogenetic plugin. To compare the Bray–Curtis distances from the D0 group to the other two groups, the Kruskal Wallis test was performed using R.⁽²²⁾

PCoA plots as 2D graphics with alpha diversity indices were shown using tidyverse ver. 1.2.,⁽²³⁾ and qiime2R ver. 0.99.13 packages of R software.^(22,23) The statistical significance of the similarity of bacterial communities among groups was assessed with the ANOSIM test using the beta-group-significance plugin.

Data availability. The nucleotide sequence dataset was deposited in the Sequence Read Archive of the DNA Data Bank of Japan under accession number DRADRR302116.

Results

Effect of Metabolokeeper® on the preservation of SCFAs in feces.

To evaluate the usefulness of Metabolokeeper®, feces from 19 healthy subjects were suspended in a preservative solution and stored at 30°C. Aliquots of the suspension were removed for the analysis of SCFAs on days 0, 7, 14, and 28 (D0, D7, D14, and D28, respectively). As a reference, an aliquot from the suspension stored at –80°C for 28 days (F28) was analyzed. Analysis data of the major SCFAs (acetic acid, propionic acid, *n*-butyric acid, iso-butyric acid, *n*-valeric acid, iso-valeric acid, and lactic acid) are presented in Supplemental Table 1*. Scatter plot analysis followed by the construction of a linear equation was performed to clarify the relationship between different groups (D0 and D7, D14, D28, and F28) and the major SCFAs, except for iso-valeric and lactic acid (Fig. 2). As shown in Table 1, the slopes (*m*) of equations for D0 vs D7, D14, and D28 were almost one (*m* = 0.9306 to 1.1889), which indicates that the SCFAs in Metabolokeeper® are very well preserved for 28 days at 30°C as well as at –80°C. No significant difference in the profile of SCFAs in each fecal specimen was observed between D0, D7, D14, and D28 (Friedman’s test, *p* > 0.05, data not shown).

Effect of Metabolokeeper® on the preservation of BAs in feces.

The test for preservation of BAs in Metabolokeeper® was performed as for SCFAs. Analysis data of the major BAs [deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), cholic acid (CA), chenodeoxycholic acid (CDCA), glycocholic acid (GCA), and 7-oxo-LCA] are presented in Supplemental Table 1*. As shown in Fig. 3, the amount of

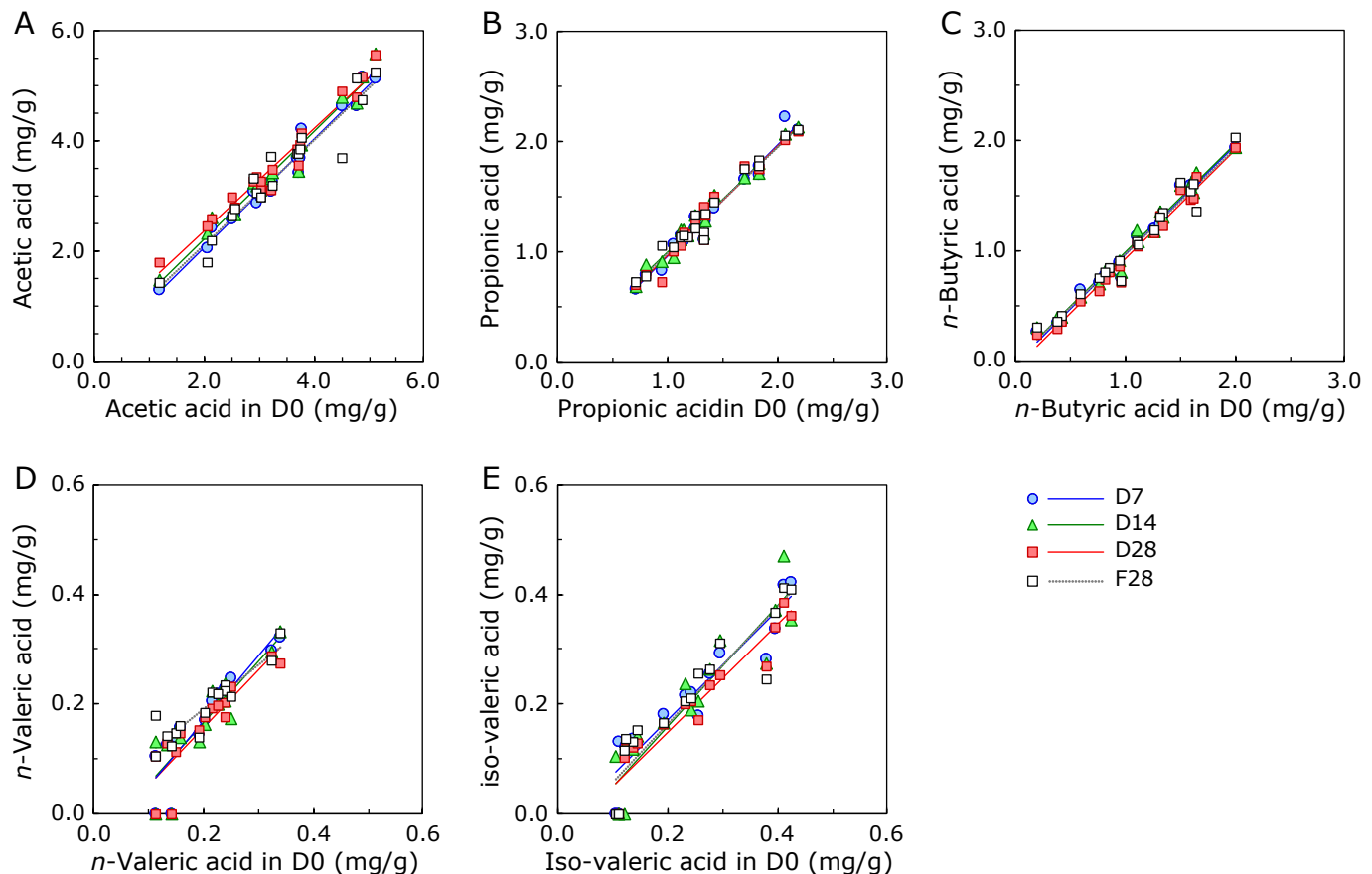


Fig. 2. Scatter plot of short-chain fatty acids from D0 and D7, D14, D28, or F28. The correlation between fecal short-chain fatty acids from D0 and D7, D14, D28, and F28 was evaluated. (A) acetic acid, (B) propionic acid, (C) *n*-butyric acid, (D) *n*-valeric acid, and (E) iso-valeric acid.

*See online. <https://doi.org/10.3164/jcfn.22-107>

Table 1. Correlation between fresh (D0) and stored (D7, D14, D28 or F28) in short-chain fatty acid

	Linear equation ($y = mx + b$)	r^2	n		Linear equation ($y = mx + b$)	r^2	n
Acetic acid				n-Valeric acid			
D0 vs D7	$y = 0.9858x + 0.0864$	0.973	19	D0 vs D7	$y = 1.1889x - 0.0689$	0.817	13
D0 vs D14	$y = 0.9763x + 0.2549$	0.973	19	D0 vs D14	$y = 1.1235x - 0.0599$	0.756	13
D0 vs D28	$y = 0.9306x + 0.4877$	0.964	19	D0 vs D28	$y = 1.0517x - 0.0538$	0.786	13
D0 vs F28	$y = 0.9501x + 0.2203$	0.924	19	D0 vs F28	$y = 0.8016x + 0.0313$	0.849	15
Propionic acid				Iso-valeric acid			
D0 vs D7	$y = 1.012x - 0.0581$	0.966	19	D0 vs D7	$y = 0.9977x - 0.0288$	0.881	15
D0 vs D14	$y = 0.9578x + 0.0372$	0.968	19	D0 vs D14	$y = 1.0924x - 0.0573$	0.859	14
D0 vs D28	$y = 0.9902x - 0.0227$	0.954	19	D0 vs D28	$y = 0.9851x - 0.0486$	0.898	14
D0 vs F28	$y = 0.9678x + 0.0203$	0.965	19	D0 vs F28	$y = 1.0628x - 0.048$	0.866	14
n-Butyric acid							
D0 vs D7	$y = 0.987x - 0.0197$	0.983	19				
D0 vs D14	$y = 0.9798x + 0.0037$	0.979	19				
D0 vs D28	$y = 0.9894x - 0.0668$	0.979	19				
D0 vs F28	$y = 0.9544x + 0.0115$	0.962	19				

Numerical datasets for iso-butyric acid and lactic acid are not presented.

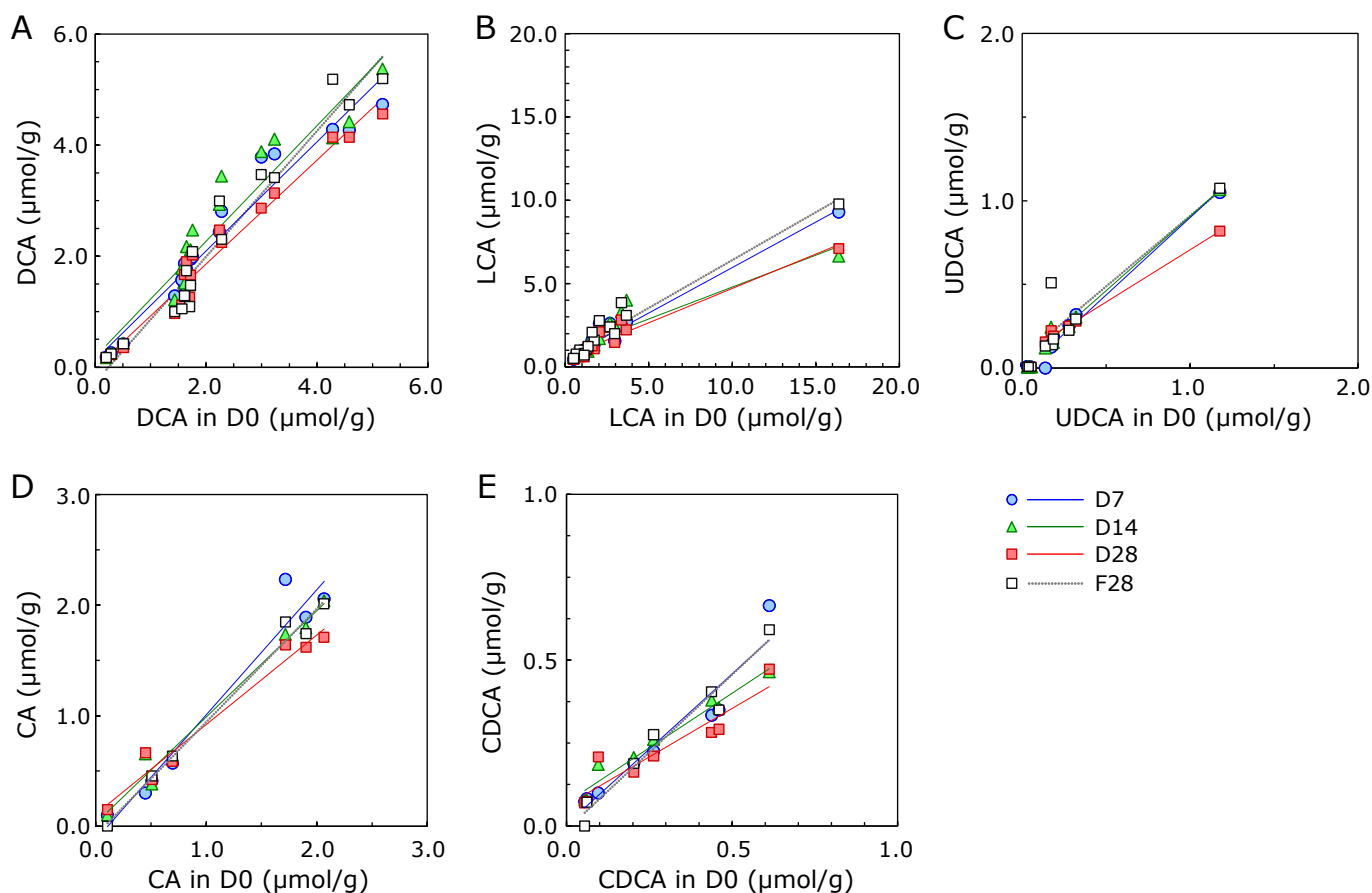


Fig. 3. Scatter plot of bile acids from D0 and D7, D14, D28, or F28. The correlation between bile acids from D0 and D7, D14, D28, and F28 was evaluated. (A) DCA, deoxycholic acid; (B) LCA, lithocholic acid; (C) UDCA, ursodeoxycholic acid; (D) CA, cholic acid; (E) CDCA, chenodeoxycholic acid. A scatter plot for glycocholic acid (GCA) or 7-oxo-lithocholic acid (LCA) is not shown.

BAs, except DCA, tended to decrease during storage. However, as shown in Table 2, the amount of each BA was well preserved during the period of 28 days for DCA ($m = 0.9347$), 14 days for UDCA ($m = 0.8901$) and CA ($m = 0.9631$), and 7 days for

CDCA ($m = 0.9080$). LCA was not well preserved even in the 7 day-storage period ($m = 0.5458$), but a high correlation was observed between D0 and D7, D14, and D28 ($r^2 = 0.830-0.967$). When subject No. 12, which had outlying LCA levels, was

Table 2. Correlations between fresh (D0) and stored (D7, D14, D28 or F28) in bile acids

	Linear equation (y = mx + b)	r ²	n		Linear equation (y = mx + b)	r ²	n
DCA				CDCA			
D0 vs D7	y = 0.9817x + 0.1280	0.949	18	D0 vs D7	y = 0.9080x + 0.0089	0.920	10
D0 vs D14	y = 1.0394x + 0.1870	0.919	18	D0 vs D14	y = 0.6606x + 0.1417	0.960	9
D0 vs D28	y = 0.9347x - 0.0131	0.963	18	D0 vs D28	y = 0.5860x + 0.1225	0.873	10
D0 vs F28	y = 1.1305x - 0.2633	0.950	18	D0 vs F28	y = 0.9375x - 0.0222	0.960	6
LCA				GCA			
D0 vs D7	y = 0.5458x + 0.5000	0.967	15	D0 vs D7	y = 0.7269x + 0.1389	0.6169	19
D0 vs D14	y = 0.3826x + 0.9571	0.830	15	D0 vs D14	y = 0.6311x + 0.1193	0.6223	19
D0 vs D28	y = 0.4119x + 0.5795	0.938	15	D0 vs D28	y = 0.7978x + 0.1717	0.5596	19
D0 vs F28	y = 0.5756x + 0.6568	0.944	15	D0 vs F28	y = 0.5980x + 0.1216	0.5871	19
UDCA				7-oxo-LCA			
D0 vs D7	y = 0.9253x - 0.0268	0.979	6	D0 vs D7	y = 6.0248x + 2.3714	0.3698	13
D0 vs D14	y = 0.8901x + 0.0167	0.989	7	D0 vs D14	y = 4.6485x + 2.3714	0.2254	13
D0 vs D28	y = 0.6260x + 0.0808	0.996	7	D0 vs D28	y = 2.7359x + 3.1154	0.0655	13
D0 vs F28	y = 0.8417x + 0.0688	0.844	7	D0 vs F28	y = 0.5414x + 0.1277	0.6206	13
CA				LCA(-No. 12)*			
D0 vs D7	y = 1.1313x - 0.1236	0.953	8	D0 vs D7	y = 0.7028x + 0.1069	0.7826	14
D0 vs D14	y = 0.9631x + 0.0245	0.982	7	D0 vs D14	y = 1.0079x + 0.0721	0.9422	14
D0 vs D28	y = 0.8104x + 0.1084	0.969	9	D0 vs D28	y = 0.6679x + 0.0958	0.8021	14
D0 vs F28	y = 1.0222x - 0.0772	0.987	6	D0 vs F28	y = 0.9178x + 0.1257	0.8162	14

*The subject No. 12 was removed from the calculation, because it had outlier in LCA. DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; GCA, glycocholic acid; 7-oxo-LCA, 7-oxo-lithocholic acid.

removed from calculations [represented as LCA(-No. 12)], the slope (m) of the linear equation increased ($m = 0.7028$). The amount of LCA also decreased in F28 ($m = 0.5756$). The correlation between D0 and D7, D14, and D28 in GCA and 7-oxo-LCA was considerably lower than that of the other BAs (Table 2), indicating that the quantified values of GCA and 7-oxo-LCA stored in Metabolokeeper® are unreliable. No significant difference in the BA profile of each fecal specimen was observed between D0, D7, D14, and D28 (Friedman's test, $p > 0.05$, data not shown). Consequently, it is recommended that the storage period in Metabolokeeper® for BA analysis be less than or equal to seven days.

Effect of Metabolokeeper® on the preservation of structure and diversity of microbiota. In this study, we evaluated the structure and diversity of fecal microbiota. As shown in Fig. 4A, there was no obvious change in gut microbiota composition at the phylum level between D0, D28, and F28. In addition, there was no obvious alteration in the gut microbiota composition at the genus level between D0, D28, and F28 (Supplemental Fig. 1*). In the analysis of microbial community structure based on Bray-Curtis distance, no significant differences in the structure of fecal microbiota were detected between the storage conditions (Fig. 4B, Supplemental Fig. 2*). In addition, no significant differences in the diversity of fecal microbiota were detected between storage conditions using α -diversity indices (Shannon, Chao1, and Simpson indices; Fig. 4C-E). These results suggest that Metabolokeeper® well preserved the fecal microbiota at 30°C and -80°C for 28 days.

Discussion

In the present study, we evaluated the usefulness of preservation using our newly developed preservation solution, "Metabolokeeper®", at 30°C for 28 days, compared to the gold standard method of immediate freezing at -80°C, on the observed microbial community structure and the metabolite

contents of fecal samples. Our results show that the new preservative successfully preserved the fecal structure of gut microbiota and fecal concentration of SCFAs, even when samples were stored at 30°C for 28 days, as well as when samples were frozen at -80°C for 28 days. BAs were successfully preserved at 30°C for 7 days only.

As the integrated analysis of gut microbiota and metabolites holds the potential to reveal interactions between the host and microbiota in relation to disease risks, the convenient and accurate simultaneous measurement of gut microbiota and metabolites in fecal samples is deeply important. Although several reports have demonstrated the stability of fecal microbiome composition,^(14,24,25) there is limited knowledge on the stability of fecal metabolites. Wang *et al.*⁽¹²⁾ have demonstrated that five fecal collection methods [immediate freezing at -20°C without preservative, OMNIgene GUT, 95% ethanol, RNAlater, and Flinders Technology Associates (FTA) cards] exhibit differences in certain measures of the gut microbiome and fecal metabolome, suggesting the importance of developing a preservative solution to stabilize fecal microbiota and organic acids such as SCFAs and BAs.

Lim *et al.*⁽¹³⁾ have described the usefulness of using OMNIgene-GUT solution to store fecal samples for the measurement of gut metabolites; however, satisfactory results were not obtained at room temperature storage. We developed a new preservative solution containing a detergent and guanidine thiocyanate. The detergent makes it possible to promptly dissolve fecal samples in the preservation solution so that more stabilization of fecal microbiota, SCFAs, and BAs at room temperature can be achieved. As the immediate deep-freezing of fecal samples is often inconvenient in routine clinical practice, this simple storage method using a preservative solution, which has an equivalent efficacy to deep-freezing, may act to stabilize fecal samples in a convenient manner.

In conclusion, this study demonstrated the usefulness of our newly developed preservation solution, "Metabolokeeper®",

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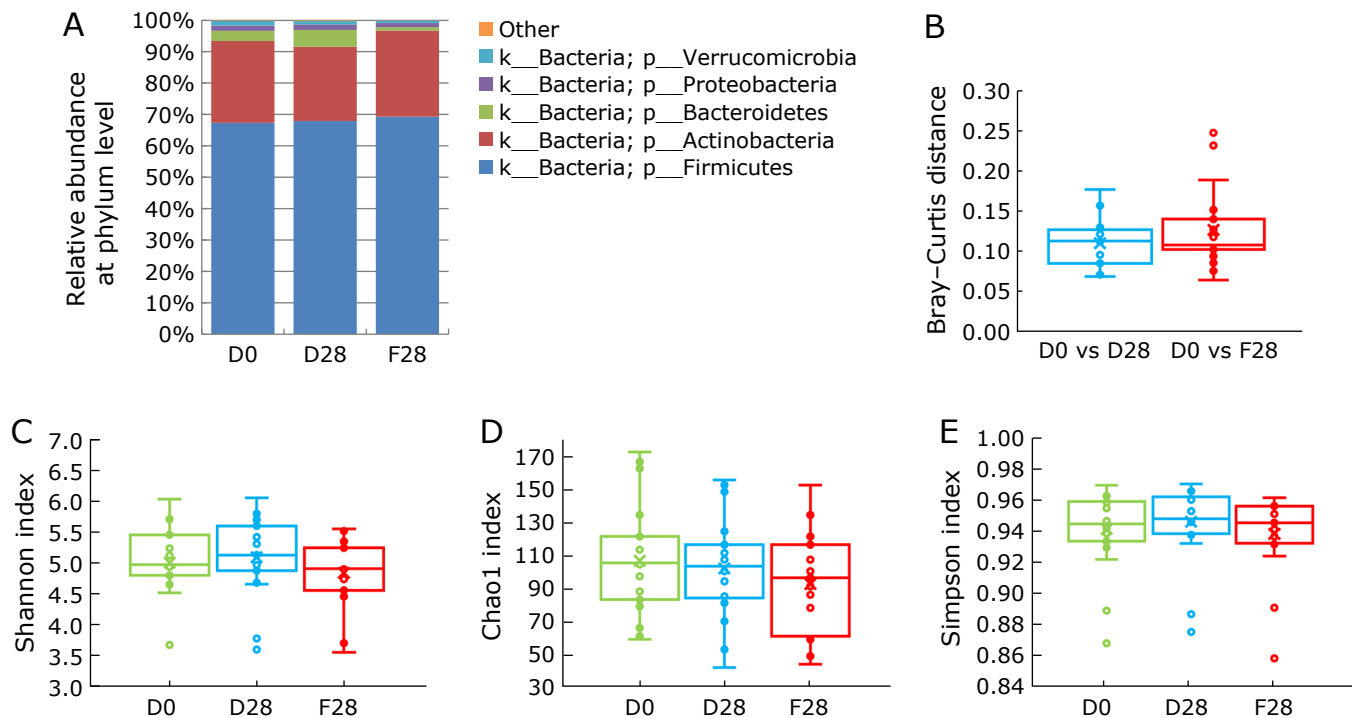


Fig. 4. Taxonomic composition and diversity of the fecal microbiota in fresh (D0) and stored (D28 or F28) fecal samples. (A) Each component of the cumulative bar chart indicates a phylum. Taxonomic composition of the fecal microbiota at phylum level was evaluated among D0, F28, and D28. (B) Microbial community structure was evaluated by Bray-Curtis distance ($p > 0.05$, Kruskal-Wallis test). The α -diversity was evaluated using Shannon (C), Chao1 (D), and Simpson (E) indices of the D0 and stored samples (D28 or F28) ($p > 0.05$, Bonferroni-Dunn test).

which does not change the structure of the gut microbiota or its metabolite levels, even when samples are left at room temperature for a long period. Although the evaluation of metabolites other than SCFAs and BAs, and the potential for longer-term preservation, with this novel solution remain unknown and further studies are warranted, this preservation solution is expected to make a great contribution to fecal collection in clinical settings, and to further our understanding of the health effects of fecal metabolites produced by the gut microbiome.

Author Contributions

Study concept and design, TT and YN; acquisition of data, TK, ST, TH, and KM; analysis and interpretation of data, TT, TK, ST, TH, and JM; drafting of the manuscript, TT and TK; critical revision of the manuscript for important intellectual content, KN; final approval, all authors; study supervision, YN.

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Abbreviations

BA	bile acid
CA	cholic acid
CDCA	chenodeoxycholic acid
DCA	deoxycholic acid
GCA	glycocholic acid
LCA	lithocholic acid
7-oxo-LCA	7-oxo-lithocholic acid
PCoA	principal coordinate analysis
SCFA	short-chain fatty acid
UDCA	ursodeoxycholic acid

Conflict of Interest

TT received collaborative research funds from Fujifilm Medical Co., Ltd. and lecture fees from Mochida Pharma. Co. Ltd., and Yanssen Pharmaceutical K.K. YN received scholarship funds from EA Pharma Co. Ltd., collaboration research funds from Taiyo Kagaku Co., Ltd., and lecture fees from Mylan EPD Co., Takeda Pharma. Co., Ltd. and Mochida Pharma. Co. Ltd., EA Pharma Co., Ltd., Otsuka Pharma. Co. Ltd. and Miyarisan Pharma. Co. Ltd. This research was not supported by any of these funds. In addition, neither the funding agencies nor any outside organizations participated in the study design nor had any competing interests.

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