Original Article

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Effects of storage temperature, storage time, and Cary-Blair transport medium on the stability of the gut microbiota

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Summary **How long fecal samples can withstand a period of refrigeration or room temperature, and the appropriate preservative, are largely unknown. Cary-Blair transport medium has been used for many years because it is inexpensive and prevents bacterial overgrowth. However, its effectiveness for metagenomic analyses has never been tested. We found that the microbial compositions using a 16S rRNA sequence of samples left at 4°C for 3 or 7 days or at 25°C for 1, 3, or 7 days differed significantly from samples stored at −80°C in no-preservative method. Whereas samples stored in Cary-Blair medium remained unchanged for longer periods. The relative abundances of phylum Bacteroidetes and Actinobacteria, changed significantly at 25°C, whereas Cary-Blair medium inhibited the reduction in Bacteroidetes and the increase in Actinobacteria. The bacterial survival counts were significantly lower in the RNAlater samples than in the Cary-Blair samples under aerobic and anaerobic culture conditions. In conclusion, storage time and storage temperature significantly affect the gut microbial composition in fecal samples. Given the low cost, inhibitory effect on bacterial changes, and potential utility in bacterial isolation, Cary-Blair medium containers are suitable for large-scale or hospital-based microbiome studies, especially if direct freezing at −80°C is unavailable.**

Keywords: Gut microbiome, Cary-Blair transport medium, RNAlater, fecal sampling, bacterial survival rate

1. Introduction

Immediate DNA extraction from fresh feces or immediate freezing at −80°C is the gold standard procedure in microbiome studies, but this can be challenging (*1-4*). To date, several preservatives have been reported (*5-8*), but most preservatives do not distinguish between DNA obtained from live or dead cells, and the bacteria are unculturable. Cary-Blair medium consisting predominantly of buffered salts with no nutrients (*9*), which suppresses bacterial overgrowth, potentially allows for long-term survival of enteric pathogens (*9*) and can be used for the isolation of

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organisms. However, the effectiveness of this medium for 16S rRNA sequencing analyses of human fecal samples has never been tested. Therefore, we examined the stability of the gut microbiota in Cary-Blair medium.

2. Materials and Methods

2.1. *Human fecal sample collection and storage methods*

Various storage methods were tested using 231 fecal samples from 11 healthy adults: (*i*) immediate DNA extraction from fresh samples (fresh); (*ii*) immediate freezing at −80°C; and (*iii*) preservation under different conditions: 4°C or 25°C for 1, 3, or 7 days (Figure 1). Microbial composition was compared between the no preservative method and Cary-Blair medium-containing method (Toyobo Co., Ltd., Tokyo, Japan) (9). Written consent was obtained from all the participants. The study was approved by the Ethics Committee of the National Center for Global Health and Medicine (No. 2014).

2.2. *Bacterial DNA extraction and sequencing 16S ribosomal RNA gene amplicons*

To extract the fecal bacterial DNA, we used an enzymatic lysis method with lysozyme (Sigma-Aldrich Co., St. Louis, MO, USA) and achromopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (*10*). The 16S amplicon PCR forward primer (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 16S amplicon PCR reverse primer (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′), with adaptor sequences for Illumina indexing, were used to amplify the bacterial 16S rRNA gene V3-V4 regions. PCRs were run for 25 cycles, using the KAPA HiFi HotStart ReadyMix PCR Kit (Nippon Genetics Co., Ltd., Tokyo, Japan). The PCR amplicons were purified with AMPure® XP magnetic purification beads (Beckman Coulter, Inc., Brea, CA, USA) and quantified with 4200 TapeStation (Agilent Technologies Japan, Ltd). Equal amounts of amplicons from all the samples were sequenced with the MiSeq System (Illumina, Inc., Tokyo Japan), according to the manufacturer's instructions (*10*).

2.3. *Bioinformatic and statistical analyses*

After the quality of the filter-passed reads with average quality values of > 25 was checked for chimeras, the taxonomy of the high-quality reads was assigned with three publically databases: the Ribosomal Database Project (RDP) v. 10.27, CORE (*http://microbiome.osu.edu/*), and a reference genome sequence database obtained from the National Center for Biotechnology Information FTP site (*ftp://ftp.ncbi.nih.gov/genbank/*, December 2011). We then selected those reads with BLAST matches of > 90% with a representative

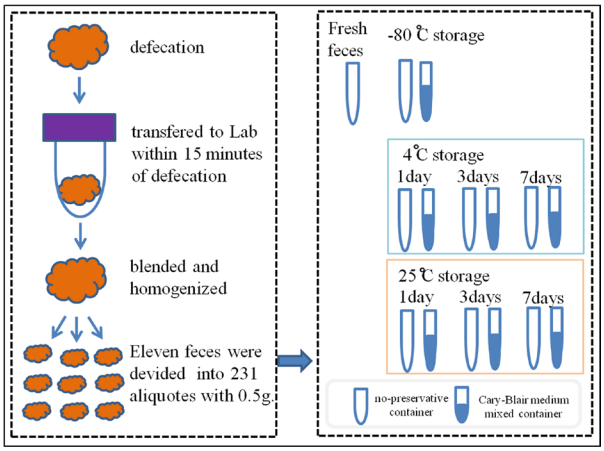

Figure 1. Sample collection and storage method.

sequence in one of the three databases. A total of 1,992,156 high-quality reads were obtained after quality filtering. We randomly selected 3,000 reads per sample and analyzed them to minimize the overestimation of the species richness in the clustering due to intrinsic sequencing error (*4,10*). Good's coverage index (*11*) of the 3,000 reads per sample in this study was 0.96, indicating high coverage, and the number of reads is sufficient for microbiome analysis. After both primer sequences were removed, the reads were sorted and grouped into operational taxonomic units (OTUs) with a sequence identity threshold of 97%. The taxonomic assignment of each OTU was made with the GLSEARCH program. Taxonomic groups with relative abundances of ≥ 0.1% in any subject were included in the subsequent analyses. All reads were deposited in the DDBJ/GenBank/EMBL database under accession number DRA 006625. Pairwise Pearson's correlation coefficients were used to analyze the microbial compositions under different storage conditions. For the UniFrac distance analyses, phylogenic-tree-based metrics were used to measure the differences in the overall bacterial compositions under different storage conditions (*12*). We defined the "reference" as the distance or coefficient between the fresh sample and those stored at −80°C. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed with the R software package (v3.2.2).

3. Results

At 4°C, weighted UniFrac distance of samples stored without preservative at 3 and 7 days was significantly larger than the reference (Figure 2A); at 25°C, the distance was significantly greater for samples at 1, 3, and 7 days (Figure 2B). In contrast, the distance for samples stored in Cary-Blair medium remained unchanged for up to 7 days at 4°C (Figure 2A) and for 24 h at 25°C (Figure 2B). Similar results were obtained at the genus level

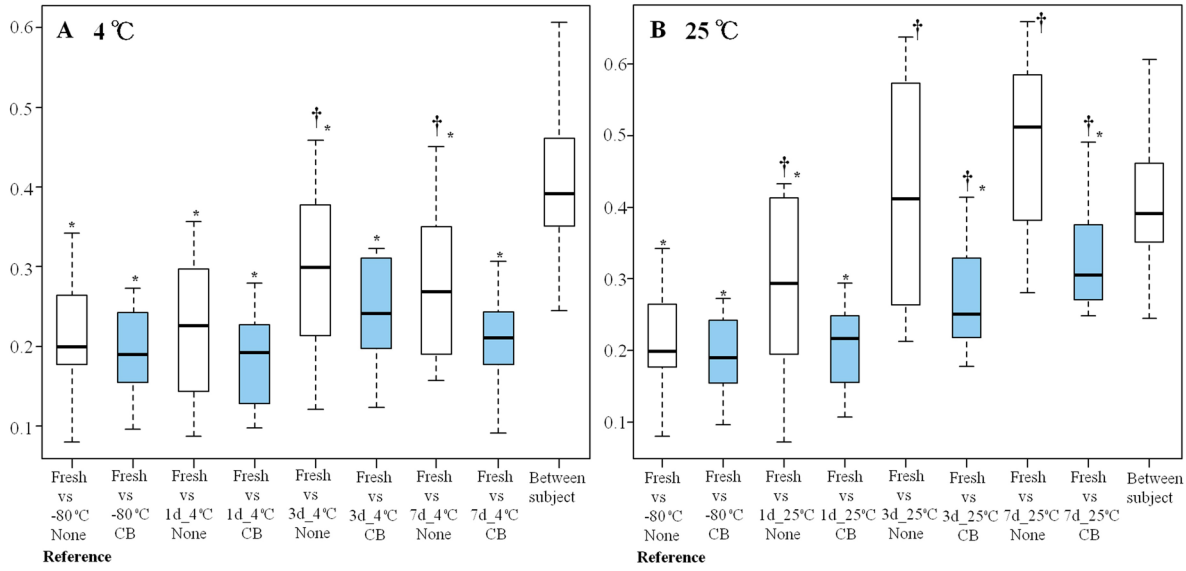

Figure 2. Box-and-whisker plot of UniFrac distances at 4°C (A) and 25°C (B) stored without preservative (white box) or in Cary-Blair (CB) medium (blue box). †$p < 0.05$ for differences between fresh samples and stored samples relative to the reference distance. *$p < 0.05$ for differences between (*i*) between-subject distances and (*ii*) within-subject distances between fresh samples and stored samples. Boxes represent the interquartile range (IQR) and lines inside show the median. Whiskers indicate the lowest and highest values within 1.5 times the IQR.

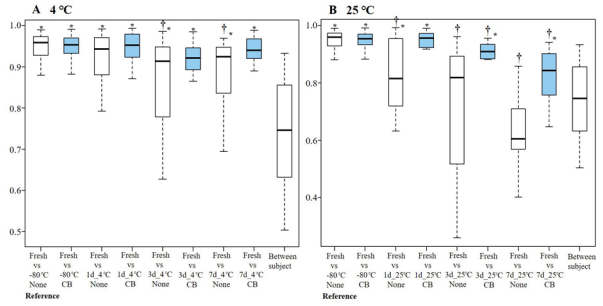

Figure 3. Box-and-whisker plot of the coefficient value at the genus level for samples stored at 4°C (A) and 25°C (B) without preservative (white box) or in Cary-Blair (CB) medium (blue box). †$p < 0.05$ for differences between fresh samples and stored samples relative to the reference distance. *$p < 0.05$ for differences between (*i*) between-subject distances and (*ii*) within-subject distances between fresh samples and stored samples. Boxes represent the interquartile range (IQR) and the lines inside show the median. Whiskers indicate the lowest and highest values within 1.5 times the IQR.

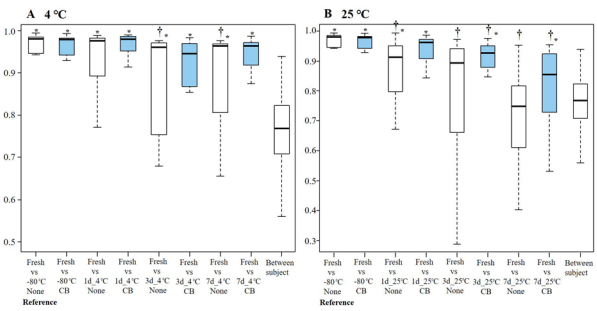

Figure 4. Box-and-whisker plot of the coefficient value at the species level for samples stored at 4°C (A) and 25°C (B) without preservative (white box) or in Cary-Blair (CB) medium (blue box). †$p < 0.05$ for differences between fresh samples and stored samples relative to the reference distance. *$p < 0.05$ for differences between (*i*) between-subject distances and (*ii*) within-subject distances between fresh samples and stored samples.

(Figure 3) and species level (Figure 4).

Relative abundance at the phylum level was significantly reduced for Firmicutes but significantly increased for Actinobacteria without preservative at 4°C at 3 and 7 days compared with the reference (Figure 5A); at 25°C, relative abundance continued to decrease for Firmicutes and Bacteroidetes from 1 day and was significantly increased for Actinobacteria at 3 and 7 days and for Proteobacteria at 1, 3, and 7 days (Figure 5B). In contrast, Cary-Blair medium inhibited the reduction in Bacteroidetes at 25°C and the increase in Actinobacteria at 3 days at 4°C and 25°C (Figure 5).

Lastly, we analyzed 9 fecal samples from 3 healthy subjects to investigate bacterial survival. We found that survival counts were significantly higher in Cary-Blair samples than in the RNAlater samples, and were not significantly different from the samples without preservative under aerobic and anaerobic culture conditions (Figure 6).

4. Discussion

This is the first study to show that the effect of Cary-Blair medium on the stability of the gut microbiomes to be used for 16S rRNA analyses. Cary-Blair medium is a nonnutritive transport medium for Gram-negative and anaerobic organisms in stool samples (*9*), and importantly, prevents the overgrowth of

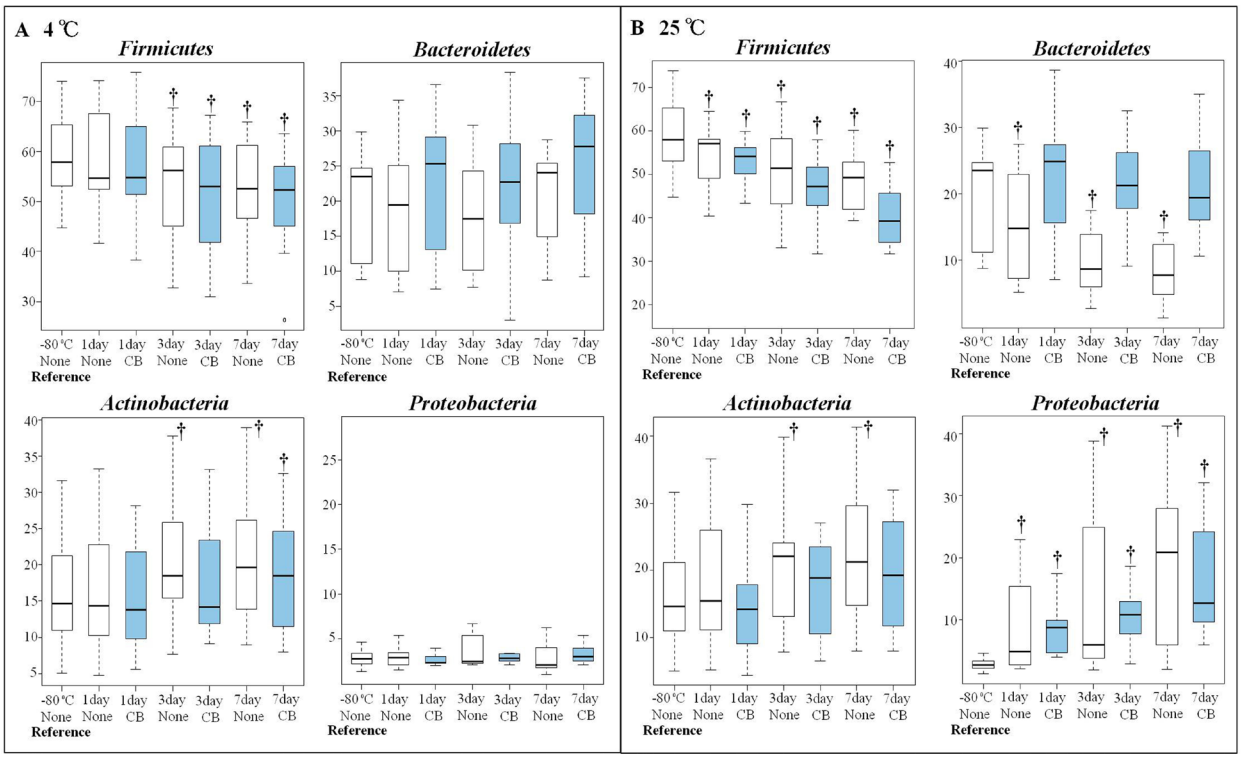

Figure 5. Time courses of relative abundance of four phyla at 4°C (A) or 25°C (B) stored without preservative (white box) or in Cary-Blair (CB) medium (blue box). *$p < 0.05$ for differences between the −80°C sample (reference) and samples stored by other methods. Boxes represent the interquartile range (IQR) and lines inside show the median. Whiskers indicate the lowest and highest values within 1.5 times the IQR.

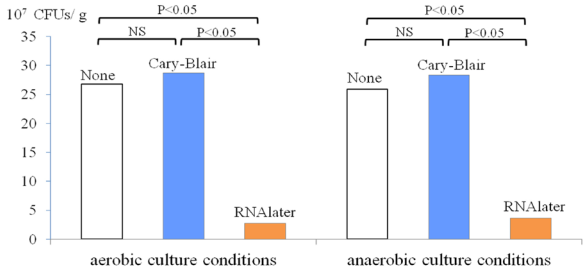

Figure 6. Bacterial survival count of fecal samples stored in Cary-Blair medium, without preservative, and in RNAlater solution under aerobic (A) and anaerobic (B) culture conditions. Survival counts were calculated as the number of colony-forming units (CFUs) and compared between fresh samples and samples stored at −80°C for 7 days. Bar chart illustrating the mean numbers of CFUs.

Enterobacteriaceae and allows the long-term survival of enteric pathogens (*13,14*). In this study, Cary-Blair medium inhibited the reduction of Bacteroidetes in samples left for up to 7 days at 25°C, and the increase in Actinobacteria in samples left for 3 or 7 days at 25°C, whereas the no-medium method did not. Consistent with these findings, Gorzelak *et al*. have shown that the detection of Bacteroidetes in no-medium fecal samples decreased after 30 min at room temperature. Therefore, one explanation of the stability of the gut microbiome in Cary-Blair medium is that it allows some bacteria to withstand longer periods at room temperature.

Another advantage of Cary-Blair medium use is that it can potentially be used for the isolation of organisms associated with specific diseases (*15*), whereas most preservatives do not distinguish between the DNA obtained from live or dead cells (*5-8*). To confirm this hypothesis, we examined the bacterial survival counts in no preservative, Cary-Blair medium, or RNAlater in the fresh and −80°C samples. We found that the survival counts were significantly lower in the RNAlater samples than in the Cary-Blair samples. In addition to this advantage, Cary-Blair medium is the least expensive of the preservatives used in microbiome studies, at less than US$0.50 dollar per container.

Our results are consistent with those of previous studies, which showed no significant change in the microbial composition of frozen fecal samples and samples stored at 4°C for up to 24 h (*3,16-18*). Few data are available regarding the effects on the gut microbiota when samples are stored at 4°C for more than 3 days. As in the present study, Choo *et al*. reported that stool storage at 4°C for 3 days significantly affected the composition of the gut microbiome compared with storage at −80°C (*5*). These results suggest that the composition of the microbiome is associated with the length of storage, even when refrigerated, and that samples should be stored at 4°C for only 3 days after defecation.

In contrast, the microbial compositions of samples left for 1, 3, or 7 days at 25°C changed substantially

compared with the reference. Consistent with this, Shaw *et al*. showed that storage for 12 h at room temperature significantly increased the weighted UniFrac distances relative to −80°C storage (*19*). Two other studies have also demonstrated a significant change in microbial composition within 3 h at room temperature (*20,21*). These findings imply that fecal samples should not be left at room temperature after defecation.

In conclusion, fecal samples without preservative should be transferred to the laboratory within 24 h of defecation when stored at room temperature or within 3 days when stored refrigerated. Given the inhibitory effect on bacterial changes, potential utility in bacterial isolation, and low cost, Cary-Blair medium containers are useful for microbiome studies, especially when direct freezing at −80°C is unavailable.

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References

1. Fouhy F, Deane J, Rea MC, O'Sullivan O, Ross RP, O'Callaghan G, Plant BJ, Stanton C. The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations. PLoS One. 2015; 10:e0119355.
2. Bahl MI, Bergstrom A, Licht TR. Freezing fecal samples prior to DNA extraction affects the firmicutes to bacteroidetes ratio determined by downstream quantitative PCR analysis. FEMS Microbiol Lett. 2012; 329:193-197.
3. Wu GD, Lewis JD, Hoffmann C, Chen YY, Knight R, Bittinger K, Hwang J, Chen J, Berkowsky R, Nessel L, Li H, Bushman FD. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol. 2010; 10:206.
4. Nagata N, Tohya M, Fukuda S, *et al*. Effects of bowel preparation on the human gut microbiome and metabolome. Sci Rep. 2019; 9:4042.
5. Choo JM, Leong LE, Rogers GB. Sample storage conditions significantly influence faecal microbiome profiles. Sci Rep. 2015; 5:16350.
6. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on. Nat Protoc. 2006; 1:581-585.
7. Song SJ, Amir A, Metcalf JL, Amato KR, Xu ZZ, Humphrey G, Knight R. Preservation methods differ in fecal microbiome stability, affecting suitability for field studies. mSystems. 2016; 1:e00021-16.
8. Roesch LF, Casella G, Simell O, Krischer J, Wasserfall CH, Schatz D, Atkinson MA, Neu J, Triplett EW. Influence of fecal sample storage on bacterial community diversity. Open Microbiol J. 2009; 3:40-46.
9. CARY SG, BLAIR EB. New transport medium for shipment of clinical specimens. I. fecal specimens. J Bacteriol. 1964; 88:96-98.
10. Kim SW, Suda W, Kim S, Oshima K, Fukuda S, Ohno H, Morita H, Hattori M. Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. DNA Res. 2013; 20:241-253.
11. Singleton DR, Furlong MA, Rathbun SL, Whitman WB. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl Environ Microbiol. 2001; 67:4374-4376.
12. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. UniFrac: An effective distance metric for microbial community comparison. ISME J. 2011; 5:169-172.
13. STUART RD. Transport medium for specimens in public health bacteriology. Public Health Rep. 1959; 74:431-438.
14. Neumann DA, Benenson MW, Hubster E, Thi-nhu-Tuan N, Le-tien-Van. Vibrio parahemolyticus in the repbulic of vietnam. Am J Trop Med Hyg. 1972; 21:464-466.
15. Lagier JC, Armougom F, Million M, Hugon P, Pagnier I, Robert C, Bittar F, Fournous G, Gimenez G, Maraninchi M, Trape JF, Koonin EV, La Scola B, Raoult D. Microbial culturomics: Paradigm shift in the human gut microbiome study. Clin Microbiol Infect. 2012; 18:1185-1193.
16. Bassis CM, Moore NM, Lolans K, Seekatz AM, Weinstein RA, Young VB, Hayden MK; CDC Prevention Epicenters Program. Comparison of stool versus rectal swab samples and storage conditions on bacterial community profiles. BMC Microbiol. 2017; 17:78.
17. Tedjo DI, Jonkers DM, Savelkoul PH, Masclee AA, van Best N, Pierik MJ, Penders J. The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. PLoS One. 2015; 10:e0126685.
18. Carroll IM, Ringel-Kulka T, Siddle JP, Klaenhammer TR, Ringel Y. Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. PLoS One. 2012; 7:e46953.
19. Shaw AG, Sim K, Powell E, Cornwell E, Cramer T, McClure ZE, Li MS, Kroll JS. Latitude in sample handling and storage for infant faecal microbiota studies: The elephant in the room? Microbiome. 2016; 4:40.
20. Cardona S, Eck A, Cassellas M, Gallart M, Alastrue C, Dore J, Azpiroz F, Roca J, Guarner F, Manichanh C. Storage conditions of intestinal microbiota matter in metagenomic analysis. BMC Microbiol. 2012; 12:158.
21. Gorzelak MA, Gill SK, Tasnim N, Ahmadi-Vand Z, Jay M, Gibson DL. Methods for improving human gut microbiome data by reducing variability through sample processing and storage of stool. PLoS One. 2015; 10:e0134802.

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