

Composition of “gold juice” using an ancient method based on intestinal microecology

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Abstract

Objective: To identify potentially effective bacterial components of gold juice, a traditional Chinese medicine treatment used for fecal microbiota transplantation.

Methods: Fecal samples were collected from five healthy children (two boys and three girls; mean age, 7.52 ± 2.31 years). The children had no history of antibiotic use or intestinal microecological preparation in the preceding 3 months. Fresh fecal samples were collected from children to prepare gold juice in mid-to-late November, in accordance with traditional Chinese medicine methods, then used within 7 days. Finally, 16S rDNA sequence analysis was used to identify potentially effective bacterial components of gold juice. QIIME software was used for comparisons of microbial species among gold juice, diluent, filtrate, and loess samples.

Results: Microflora of gold juice exhibited considerable changes following “ancient method” processing. Microbial components significantly differed between gold juice and filtrate samples. The gold juice analyzed in our study consisted of microbes that synthesize carbohydrates and amino acids by degrading substances, whereas the filtrate contained probiotic flora, *Bacteroides*, and *Prevotella* 9.

Conclusions: This study of microbial components in gold juice and filtrate provided evidence regarding effective bacterial components in gold juice, which may aid in clinical decisions concerning fecal microbiota transplantation.

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Keywords

Gold juice, filtrate, bacteria components, 16S rDNA, fecal microbiota transplantation, Prevotella, Bacteroidetes, probiotics, traditional Chinese medicine, sequence analysis

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Introduction

Human fecal transplantation has been performed in China since at least the Eastern Jin Dynasty (266-420). A Chinese traditional medicine doctor, Ge Hong, described the symptoms of “typhoid fever and timely temperature disease...lasting six or seven days...patients feel acute heat, fainting, fatigue, and are almost dying.”¹ The treatment was “twisted fecal juice, combined with one or two liters of water (huanglong decoction)... when stored for a longer duration, it has better efficacy.”¹ This is considered the origin of fecal microbiota transplantation (FMT) in China.² Later generations of doctors processed feces into “gold juice,” which has heat-clearing and detoxifying effects, is widely used in treatment of febrile diseases, and exhibits a good clinical effect.³⁻⁵ In recent years, studies regarding gold juice have mainly focused on literature discussion and/or comparison of modern FMT methods,⁶⁻⁹ but modern in-depth studies have not been widely performed, which limits the clinical application of gold juice.

The healthy adult gut contains >220 genera, with $>10^{11}$ cells per gram of large intestinal contents.^{10,11} These microbes are present in specific quantities and proportions among the various intestinal segments; their distributions within the gastrointestinal tract are presumed to maintain symbiotic or antagonistic relationships, thus ensuring ecological balance. The microbes contribute to the host's nutrition, metabolism, and absorption; they also impact the host's immune and physiological

functions.¹² However, these microbial communities are not fully understood, particularly in the context of FMT. 16S rDNA sequence analysis enables more detailed investigations of FMT, compared with traditional microbial culture methods; this sequence analysis approach is easy, rapid, accurate, and sensitive. Thus, it has been widely used in the study of intestinal flora.¹³ Gold juice is derived from feces and may use intestinal flora for disease treatment. Therefore, 16S rDNA sequence analysis was used in this study to identify potentially effective bacterial components of gold juice. Based on the use of gold juice in the traditional Chinese medicine approach to FMT, the findings in this study might aid in clinical analyses of FMT and elucidation of the treatment mechanisms.

Materials and methods

Fecal sample collection

This study protocol was approved by the Medical Ethics Committee of Tianjin Second People's Hospital. All participants and their parents provided verbal informed consent. Fecal samples were collected from five healthy children (two boys and three girls; mean age, 7.52 ± 2.31 years). Fecal donors met the following criteria: (1) all were healthy children (i.e., they had no diagnosis of illness or disability), 6 to 14 years old; (2) who had had not used antibiotics in the preceding 3 months; (3) nor had used an intestinal microecological preparation in the preceding 3 months; (4) nor

had gastrointestinal tumors, Crohn's disease, ulcerative colitis, constipation, other digestive diseases, or digestive system symptoms; (5) nor had a history of immune system diseases or use of immunosuppressive agents; (6) nor had traveled to a region with locally widespread diarrhea in the preceding 6 months; and (7) nor had a history of infectious diseases in the preceding 3 months. All fresh fecal samples were collected by a routine clinical method (i.e., by sitting on a bucket with a collecting device placed underneath) within a 7-day period in mid-to-late November, in accordance with traditional Chinese medicine methods. They were then stored in a 4°C refrigerator for further analysis.

Gold juice preparation

The method used to prepare gold juice followed the approach described in several published texts.^{14–17} The collected feces from all donors were combined in a disinfected bucket and diluted with distilled water at a ratio of 1:6 to obtain the fecal diluent. The filter screen was constructed using sterile gauze (Henan Medical Equipment Company, Zhengzhou, China) and loess (obtained from Chifeng, China), and the diluent was filtered to remove the visible component. The filtrate was divided into ceramic jars (obtained from Chengdu, China); the jars were sealed with a mixture of 0.9% saline solution and yellow clay (obtained from Tianjin, China). The filtrate was buried 1 m underground to avoid exposure to sunlight. The supernatant obtained in the jar was regarded as “gold juice” when the jar was harvested approximately 2 years later.

Filtrate preparation

Filtrates were prepared in accordance with the method described above, and the diluent from the production process was

separated into three aliquots when feces were mixed with water (2.5 mL each). The loess from the production process was separated into three pieces (1 g each). Filtrate from the production process was separated into three aliquots (2.5 mL each). Fifteen liters of filtrate were divided equally into three jars to prepare the gold juice. Two years later, three aliquots of gold juice supernatant were collected (2.5 mL each; one aliquot per jar). Concurrently, liquid in the middle and lower layers of each jar (i.e., “lower liquid”) was collected in three aliquots (2.5 mL each). All of the above specimens were stored at –80°C immediately, then sent to Shanghai Ouyi Biomedical Technology Co., Ltd. (Shanghai, China) for 16S rDNA high-throughput Illumina MiSeq sequencing.

Main reagents and instruments

The following key reagents and instruments were used for sequencing analysis in this study: hexadecyl trimethyl ammonium bromide (synthesized by Shanghai Ouyi Biomedical Technology Co., LTD.); Qubit dsDNA Assay Kit (cat. no. Q328520; Life Technologies, Carlsbad, CA, USA); HiFi Hot Start Ready Mix hi-fi enzyme (cat. no. KK2501; Kapa Biosystems, Wilmington, MA, USA); centrifuge, model 5418 (Eppendorf, Hamburg, Germany); PCR instrument, model 580BR10905 (Bio-Rad, Hercules, CA, USA); electrophoresis instrument, model he-120 (Tanon, Shanghai, China); gel imager, model 2500 (Tanon); pipettes (Eppendorf); Agilent 2100 Bioanalyzer chip analysis system (Agilent, Santa Clara, CA, USA); and Illumina MiSeq high-throughput sequencing platform (Illumina, San Diego, CA, USA).

Sequencing analysis

Samples were thawed at room temperature, after removal from the –80°C freezer;

DNA extraction and PCR amplification were then performed at room temperature. Genomic DNA was extracted using hexadecyl trimethyl ammonium bromide, and the purity and concentration of DNA were detected by agarose gel electrophoresis and an Agilent 2100 Bioanalyzer. Samples were then diluted to 1 ng/ μ L with sterile water. Using diluted genomic DNA as the template, specific primers with barcodes were used to amplify the target regions. The forward primer was 338f: 5'-ACTCCTACGGGAGGCAGCA-3'; the reverse primer was 806r: 5'-GGACTACHVGGGTWTCTAAT-3'. PCR was performed using HiFi Hot Start Ready Mix. The PCR protocol was as follows: initial denaturation at 94°C for 5 minutes; 27 cycles of denaturation at 94°C for 30 s and elongation at 72°C for 30 s; and a final extension at 72°C for 10 minutes. PCR product purification was performed with electrophoresis and magnetic beads at Shanghai Ouyi Biomedical Technology Co., Ltd., using a proprietary method. After purification, the PCR product was used as a template for nested PCR; nested PCR was performed at Shanghai Ouyi Biomedical Technology Co., Ltd., using a proprietary method. Equal volumes of samples were then used in sequencing reactions, in accordance with the sequencing reagent manufacturer's instructions (Illumina).

Statistical analysis

Trimmomatic software was used to conduct hybridization of the original double-ended sequence to remove ambiguous base, single-base homologous, and excessively short sequences. The parameters for accurate impurity removal were as follows: remove sequences containing N bases and retain sequences with the Q20 base mass fraction of $\geq 75\%$. Usearch software was used to detect and remove chimeric sequences. Sequencing data were preprocessed to

generate high-quality sequences; UPARSE software was then used to classify sequences into multiple operational taxonomic unit (OTU) sequences based on their similarity. The parameter of $\geq 97\%$ sequence similarity was used to define an OTU. The QIIME software package was used to select representative sequences for each out; all representative sequences were compared with Greengenes reference data for annotation. The species comparison annotation used RDP classifier software, and annotation results with confidence intervals >0.7 were retained. All above software and analysis methods used in this study were obtained from original sources and customized by Shanghai Ouyi Biomedical Technology Co., Ltd. Statistical analyses in our laboratory were performed using SPSS Statistics, version 13.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Changes in biological diversity of gold juice, filtrate, diluent, and loess

OTUs were established based on $\geq 97\%$ sequence similarity with standardized sequences; 23,376 unique sequences were identified. The following methods were used to construct cluster analysis charts (Figure 1): weighted UniFrac distance (based on evolutionary relationships and species abundance), principal coordinate analysis (PCoA) of each category, and overall similarity according to the unweighted pair group method with arithmetic mean (UPGMA) method.¹⁸ PCoA analysis (Figure 1a) revealed that the samples were strongly clustered into separate bacterial communities, such that PC1 and PC2 explained 77.15% and 17.26% of the changes, respectively. The microbial species identities and abundances in gold juice were significantly different ($P < 0.05$) from those

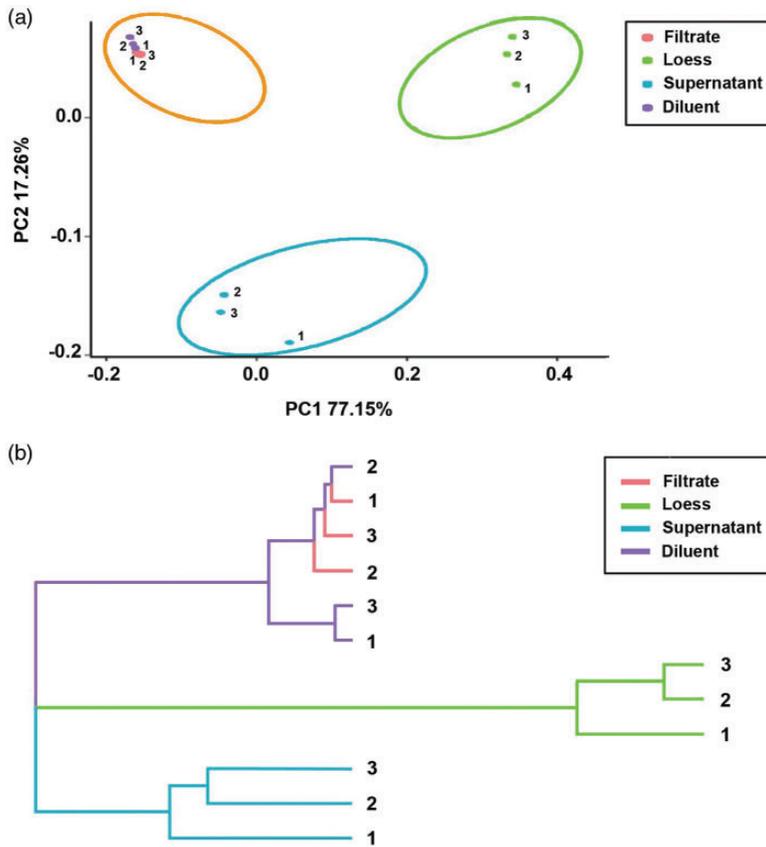


Figure 1. β -diversity analysis of different groups. (a) PCoA analysis based on weighted UniFrac distance. (b) Clustering analysis using Unweighted Pair Group Method through Arithmetic means (UPGMA) method.

in diluent, filtrate, and loess samples. The microbial species identities and abundances in diluent and filtrate samples were significantly different ($P < 0.05$) from those in loess samples, whereas there were no significant differences in microbial species identities or abundances between filtrate and diluent samples. Although the microbial compositions were similar between diluent and filtrate samples, they did not completely coincide (Figure 1b). This finding indicated that, although the samples were exposed to loess microflora during the gold juice production process, a relatively small amount of microflora was introduced into the filtrate, resulting in minimal changes in

microflora between filtrate and diluent samples. However, after the completion of “ancient method” processing, the microflora of gold juice was considerably different from that of other samples taken during the production process.

Comparison of visual and olfactory properties between gold juice and filtrate

The filtrate exhibited a suspension appearance with no floating objects on it, such that it comprised yellow mud and water with a foul odor. The gold juice constituted a golden solution, which was clear, transparent, and free of foul odor (Figure 2).

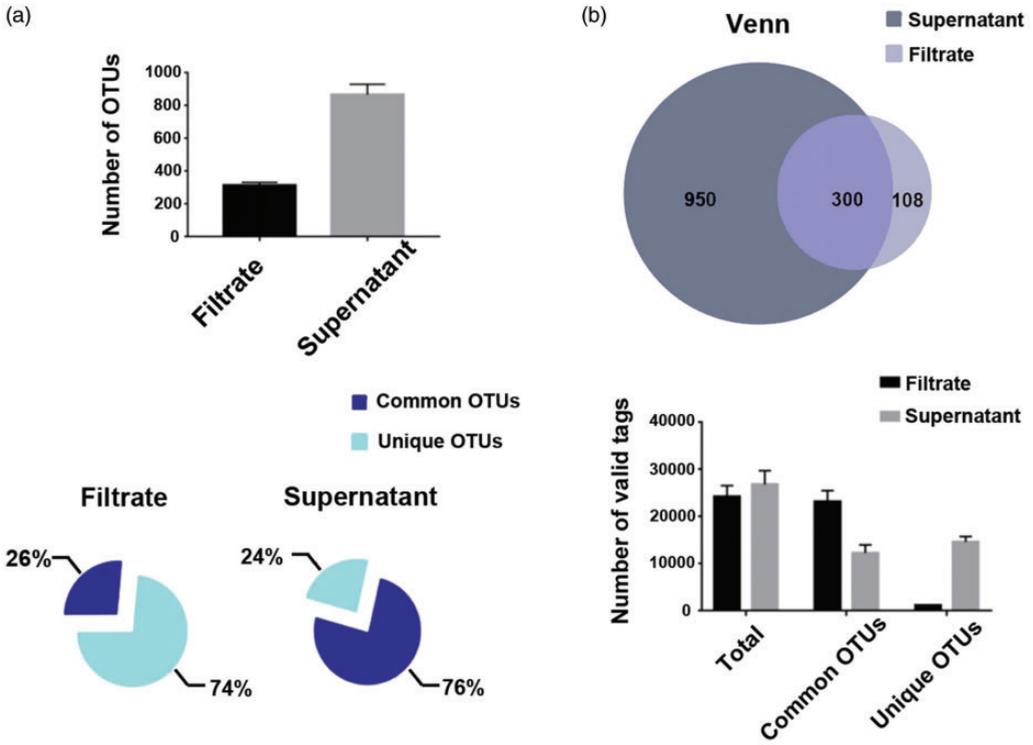


Figure 2. Visual comparison of filtrate and gold juice.

This was consistent with the traditional Chinese medicine literature: “if the water is clear, there is no foul air”.¹⁹

Comparison of microbial composition between gold juice and filtrate

After gold juice and filtrate had been sequenced, hybridization, chimeric removal, and post-processing were performed. Valid tag data were determined in each sample, and OTU classification was carried out as described in the Methods.²⁰ The mean numbers of valid tags in gold juice and filtrate were 26,761.33 and 24,196.00, respectively; these numbers did not significantly differ. There were 866.33 OTUs in gold juice alone and 314.33 OTUs in filtrate alone ($P < 0.01$; Figure 3a). There were 300 OTUs shared by both gold juice and

filtrate; the mean number of valid tags was 14,556.67 ($P < 0.01$). There were 950 OTUs specific to gold juice (76%) with 1108.33 mean valid tags, whereas there were 108 OTUs specific to filtrate (26%) with 12204.67 mean valid tags; these findings significantly differed between samples ($P < 0.01$; Figure 3b).

Structures of microbial communities

Based on the differences in OTUs between gold juice and filtrate, the numbers of representative OTUs in each sample (and their proportions) were compared by SPSS software analysis; representative sequences were annotated through a naive Bayesian classification algorithm using the RDP classifier.²¹ Subsequently, histograms were generated to compare the relative abundances of species to observe changes in community

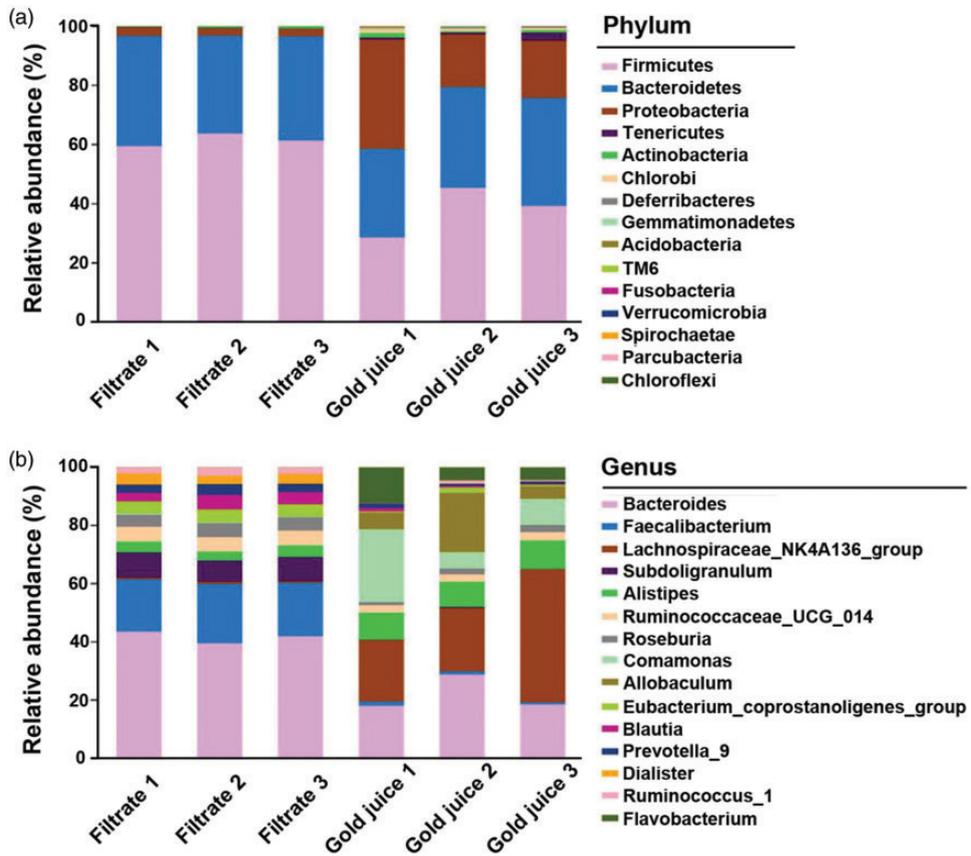


Figure 3. Comparison of OTUs between gold juice and filtrate. (a) Effective tag histogram based on OTU classification. (b) Venn diagram based on OTU classification.

structure at the phylum and genus levels (Figure 4).

At the phylum level, Firmicutes (37.80%), Bacteroidetes (33.38%), Proteobacteria (24.67%), Tenericutes (1.38%), and Chlorophyta (0.92%) were the main bacteria present in gold juice. In contrast, Firmicutes (61.62%), Bacteroidetes (35.10%), Proteobacteria (2.65%), soft Firmicutes (0.45%), and Actinomycetes (0.16%) were the main bacteria present in filtrate. The abundances of bacteria in gold juice and filtrate were clearly distinct; however, Bacteroidetes, Firmicutes, and Proteobacteria were the

dominant types of bacteria in both samples. Further analyses revealed that the abundance of Firmicutes decreased by 38.65%, the abundance of Proteobacteria increased by 8.31-fold, and the abundance of Bacteroidetes did not significantly change in gold juice, compared with filtrate (Figure 4a). In gold juice, the abundances of the genera changed in each phylum, relative to filtrate (Figure 4b). The Lachnospiraceae_NK4A136_group (Firmicutes, 8.71%), *Bacteroides* (Bacteroidetes, 6.74%), *Allobaculum* (Firmicutes, 3.49%), *Trichomonas plexus* (Proteobacteria, 3.32%), and *Alistipes* (Bacteroidetes,

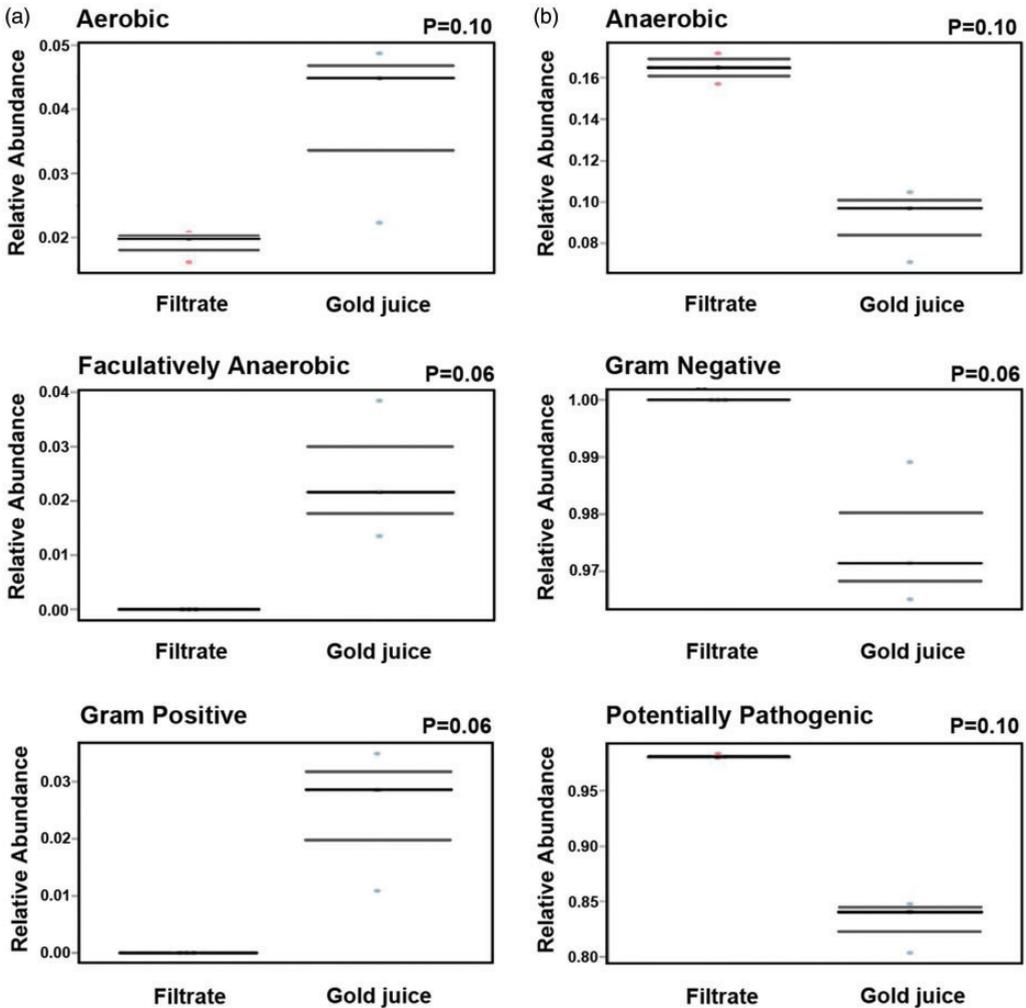


Figure 4. Microbial community distributions in gold juice and filtrate. (a) Abundance at phylum level, excluding first 15 species from histograms. (b) Abundance at genus level, including the first 15 representative species from histograms. Species with larger areas in figure are dominant species.

2.67%) were dominant in gold juice. In contrast, filtrate mainly include *Bacteroides* (Bacteroidetes, 29.90%), *Faecalibacterium* (Firmicutes, 13.50%), *Subdoligranulum* (Firmicutes, 5.87%), *Ruminococcaceae* UCG 014 (Firmicutes, 3.60%), *Roseburia* (Firmicutes, 3.37%), *Dialister* (Firmicutes, 2.44%) and *Prevotella* 9 (Firmicutes, 2.22%).

Analyses of bacterial phenotypes

Phenotypic analyses were performed using the 16S Greengenes database, KEGG database, and functional gene annotation with BugBase,²² followed by the Mann–Whitney–Wilcoxon test (Figure 5; Table 1). The relative abundance of aerobic bacteria increased in gold juice, relative to filtrate;

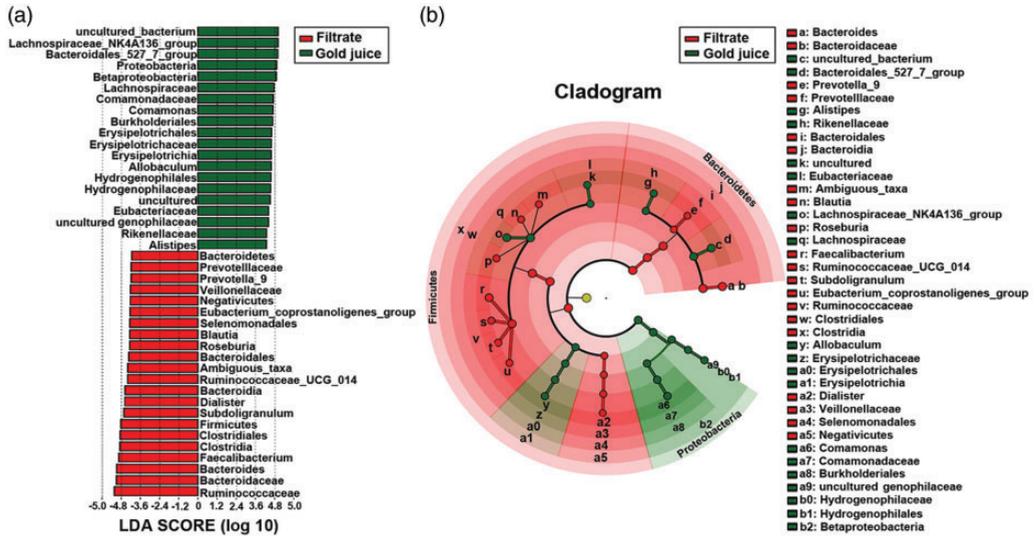


Figure 5. Analyses of bacterial phenotype. Figure shows relative abundances of bacterial communities in gold juice and filtrate in terms of aerobic, anaerobic, facultative anaerobic, gram-negative, gram-positive, and pathogenic phenotypes. P values were calculated using Mann–Whitney–Wilcoxon test; $P < 0.05$ was considered to indicate a significant difference. Each set of three lines represents (from top of graph to bottom of graph) upper quartile, average, and lower quartile, respectively.

Table I. Relative abundances, according to bacterial phenotype.

Phenotype	Filtrate abundance	Gold juice abundance	P value
Aerobic	1.89% ± 0.24%	4.49% ± 1.43%	0.10
Anaerobic	16.46% ± 0.74%	9.09% ± 1.77%	0.10
Facultatively anaerobic	0.00% ± 0.00%	2.45% ± 1.27%	0.06
Gram-negative	100.00% ± 0.00%	97.52% ± 1.25%	0.08
Gram-positive	0.00% ± 0.00%	2.48% ± 1.25%	0.06
Potentially pathogenic	98.26% ± 0.00%	83.06% ± 2.37%	0.10

however, this difference was not statistically significant. The relative abundance of anaerobic bacteria decreased in gold juice, relative to filtrate; this difference was also not statistically significant. The relative abundance of facultative anaerobic bacteria was greater in gold juice than in filtrate; however, this difference was not statistically significant. The relative abundances of Gram-negative bacteria in gold juice and filtrate were both high; these abundances did not significantly differ. The relative

abundances of Gram-positive bacteria were both low; these abundances did not significantly differ. The abundances of opportunistically pathogenic bacteria were lower in gold juice than in filtrate; however, these abundances did not significantly differ. Overall, gold juice and filtrate both contained mainly anaerobic bacteria, with smaller proportions of aerobic and facultative anaerobic bacteria. Gram-negative bacteria were predominant, while smaller numbers of Gram-positive bacteria were

also present. There were no differences in pathogenicity between the two types of samples.

Analyses of bacterial flora specific to gold juice

Using the union set of bacteria with mean relative abundances of >2% in both gold juice and filtrate, the linear discriminant analysis effect size was calculated, and the Kruskal–Wallis rank-sum test was used to identify representative flora with significant differences in abundance between gold juice and filtrate (Figure 6).²³ Thirty-nine

representative species were identified (Figure 6a). *Alistipes* and *Bacteroidales_527_7_group* were present in both gold juice and filtrate groups; the *Lachnospiraceae_NK4A136_group*, *Allobaculum*, and an uncultured bacterial family differed in abundance between gold juice and filtrate. The abundances of *Comamonas* and uncultured *genophilaceae* were higher in gold juice than in filtrate ($P < 0.05$). The following bacteria were less abundant in gold juice than in filtrate: the phyla *Bacteroidetes* and *Firmicutes*; the family *Lachnospiraceae*; the genera *Prevotella* 9, *Blautia*, *Roseburia*, *Faecalibacterium*, and

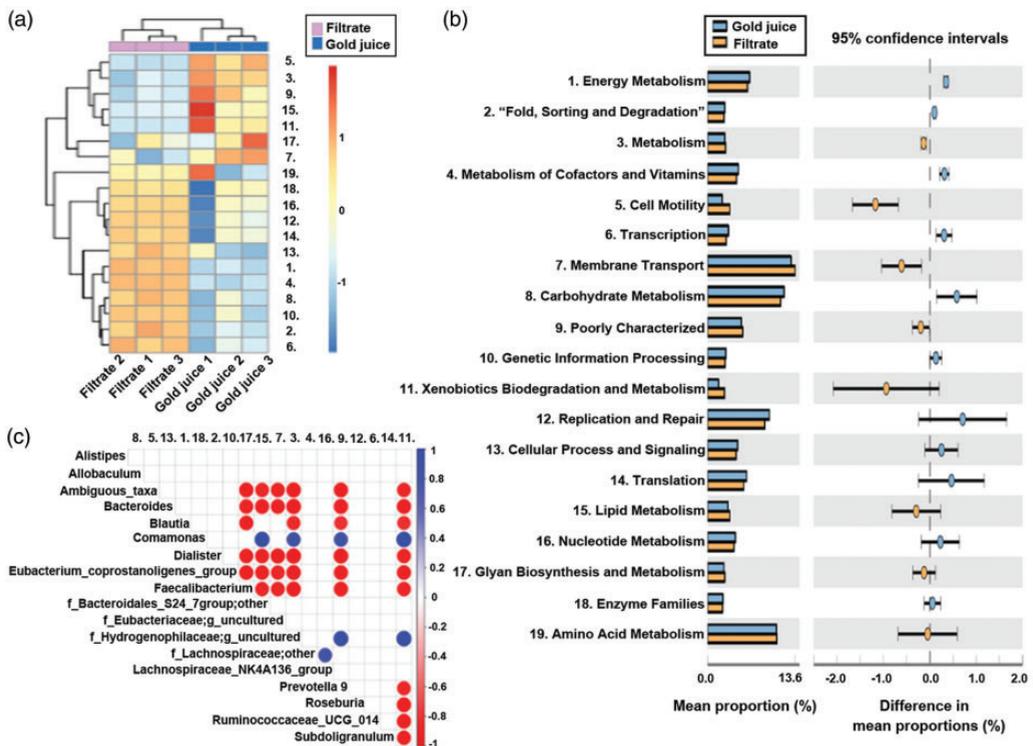


Figure 6. Linear discriminant analysis effect size analysis of gold juice and filtrate. (a) Histogram of distribution of linear discriminant analysis values shows species with significant differences in relative abundance between gold juice and filtrate; lengths of histogram bars represent influences of indicated species. (b) Evolutionary branch figure shows differences between species in gold juice and filtrate, as well as evolution of biomarker diagram. Yellow nodes indicate no significant differences between groups, red nodes indicate distinct bacterial taxa in filtrate, and green nodes indicate distinct bacterial taxa in gold juice. Abbreviation: LDA, linear discriminant analysis.

Subdoligranulum; the species *Eubacterium coprostanoligenes*; and the ambiguous taxon Ruminococcaceae UCG. The abundances of *Dialister* and Veillonellaceae were also lower in gold juice than in filtrate ($P < 0.05$; Figure 6b).

Prediction of metabolic functions of gold juice

Based on the results of 16S rDNA sequencing, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was used to predict bacterial community function by comparison with the Greengenes database. The microbial functions were distinct between samples (Figure 7a), indicating that the metabolic functions of microflora differed between gold juice and filtrate. Student's t-test was used to compare PICRUSt findings between gold juice and filtrate (Figure 7b). Membrane transport, carbohydrate metabolism, replication and repair, and amino acid metabolism were prominent functions for bacterial communities in both gold juice and filtrate. Gold juice exhibited robust functions in the following categories, compared with filtrate ($P < 0.05$): energy metabolism, "folding, sorting and degradation," transcription, metabolism of coenzyme factors and vitamins, carbohydrate metabolism, and genetic information processing. Filtrate exhibited robust functions in the following categories, compared with gold juice ($P < 0.05$): cell motility, metabolism, membrane transport, and poorly characterized. Finally, Pearson correlation analysis was conducted between flora and the metabolic pathways identified by PICRUSt analysis. Notably, there were no positive or negative correlations between the flora and main metabolic pathways (Figure 7c).

Comparison of gold juice with lower liquid

Weighted UniFrac distances were analyzed by PCoA, which revealed that microbial compositions differed between gold juice and filtrate. Specifically, PC1 explained 81.32% of the change, PC2 explained 10.79% of the change, and PC3 explained 6.44% of the change (Figure 8a). Species annotation was performed using the representative OTUs and compared with the reference database; comparisons of relative species abundances were carried out using the Kruskal–Wallis test. The combined abundances of Firmicutes, Bacteroidetes, and Proteobacteria in both gold juice and lower liquid were $>90\%$ (Figure 8b). At the phylum level, the phyla Tenericutes, Deferribacteres, and Firmicutes were more abundant in gold juice than in lower liquid ($P = 0.0369, 0.0463, \text{ and } 0.0495$, respectively) (Figure 8d). In contrast, Spirochaetae, Acidobacteria, Nitrospirae, Actinobacteria, Hydrogenedentes, Fibrobacteres, and Proteobacteria were less abundant in gold juice than in lower liquid (all $P = 0.0495$); the abundance of Bacteroidetes did not differ between sample types. At the genus level, *Alistipes*, *Bacteroides*, and Lachnospiraceae_NK4A136_group were more abundant in gold juice than in lower liquid (all $P < 0.05$). The abundances of *Proteiniphilum*, *Flavobacterium*, *Azospirillum*, and *Comamonas* were higher in lower liquid than in gold juice (all $P < 0.05$; Figure 8c).

Discussion

FMT has been shown to replace or strengthen the intestinal microflora of patients with various diseases, using intestinal microflora from healthy donors.^{24–27} In 2013, the first randomized, controlled trial using FMT for recurrent *Clostridium difficile* infection was reported; it demonstrated greater therapeutic efficacy than

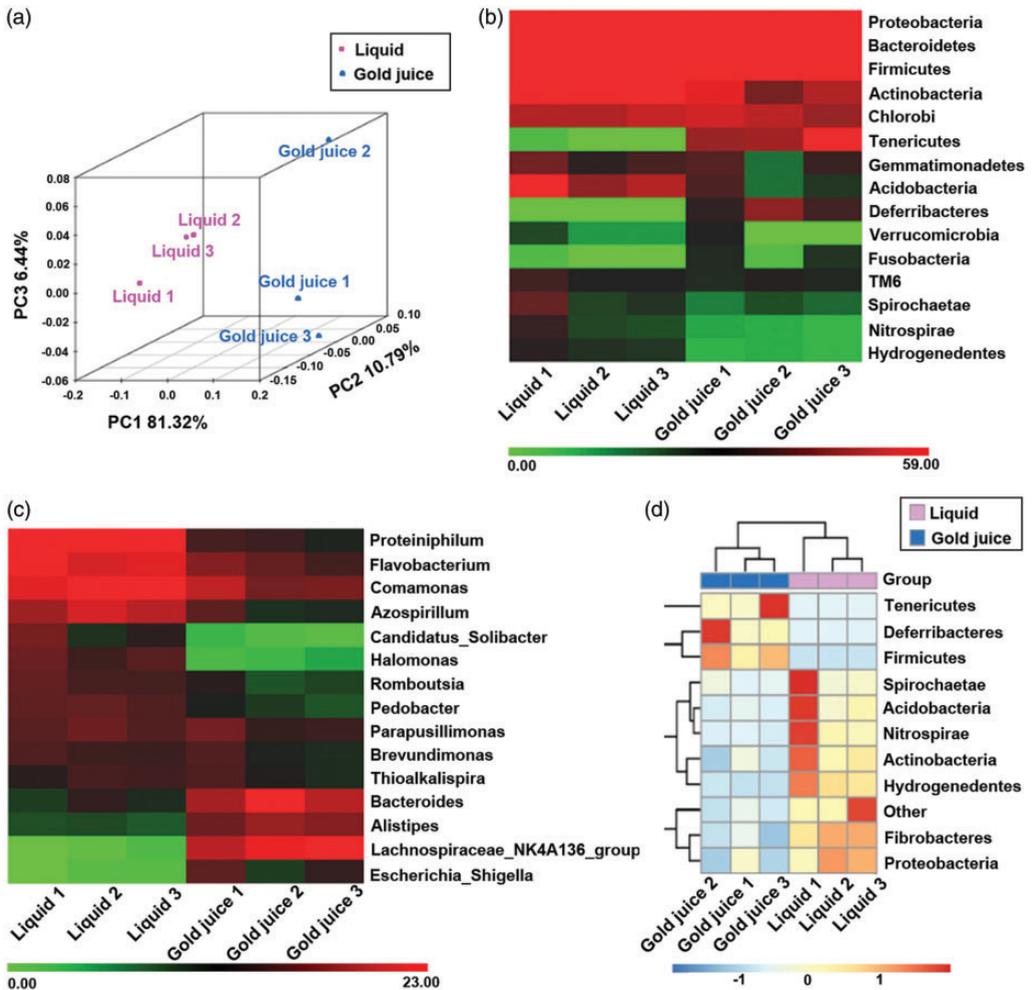


Figure 7. Predicted metabolic function according to differences in microflora. (a) Heat map based on KEGG analysis. (b) KEGG pathways were compared between groups using Student’s t-test; significant differences are shown, where blue represents gold juice and orange represents filtrate. Bar chart on left represents number of reads enriched in KEGG analysis, with corrected P value on right. (c) Analysis of correlations between flora and metabolic pathways: red indicates negative correlation, while blue indicates positive correlation.

vancomycin.²⁸ In the same year, the US Food and Drug Administration approved the use of human feces in the treatment guidelines for recurrent *C. difficile* infection. Subsequently, FMT has continued to flourish, with a wider range of indications.^{29–34} With the progressive development of FMT, its limitations have become

clearer; these limitations are as follows.^{35,36} 1) For the safety of patients undergoing FMT, strict expert consensus guidelines are used to guide donor selection; the process is complex and involves considerable effort (e.g., health and medical screening, especially regarding a history of temporary or permanent intestinal dysbacteriosis;

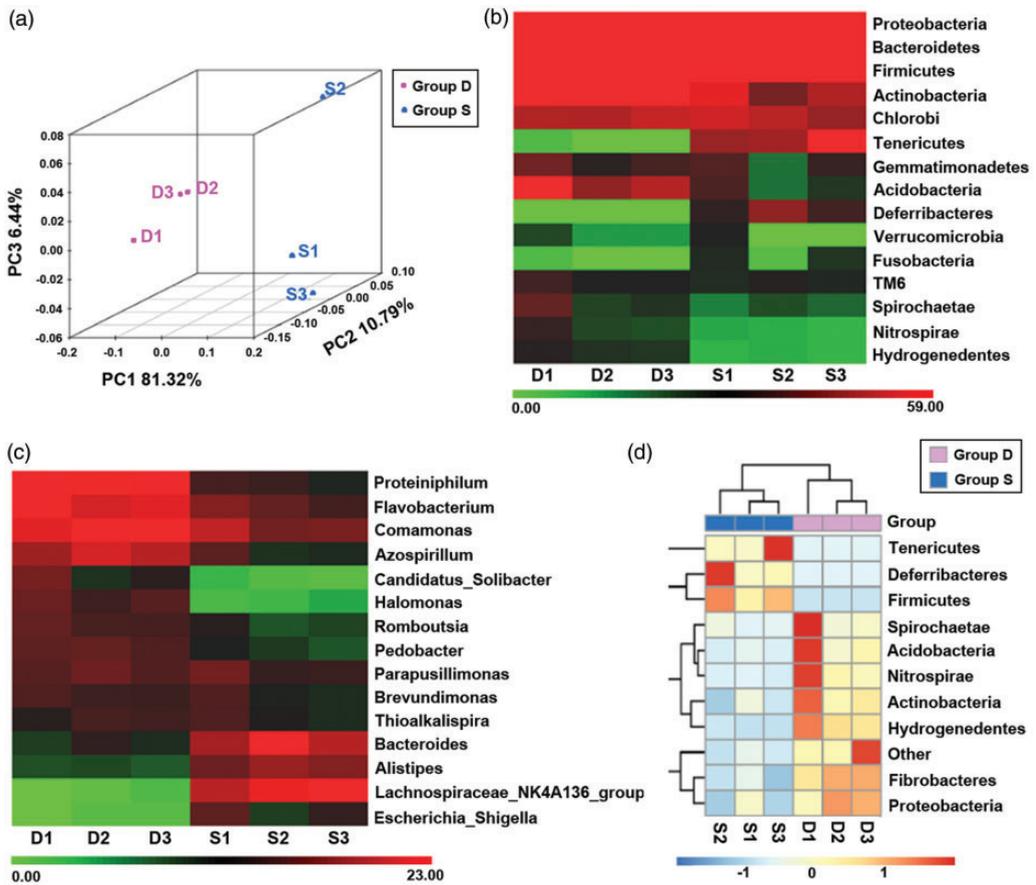


Figure 8. Comparison of gold juice with lower liquid. (a) PCoA analysis based on weighted UniFrac distance. (b) Heat map of top 15 species at phylum level. (c) Heat map of top 15 species at genus level. (d) Kruskal–Wallis test results for species classification, with abundance heat map of different species at phylum level.

serologic screening; and conventional detection.³⁷ Despite these stringent guidelines, failure may occur after screening of collected waste. 2) Donor selection is strict, so obtained feces are valuable. Beginning in 2012, Openbiome in the US, Advancing Bio, France University Hospitals of Paris Center, Taymount Clinic in Britain, Netherlands Donor Feces Bank, and FmtBank³⁸ have been opened to ensure the quality of source feces and bacteria used in FMT treatment. Although current studies have shown no differences in

efficacy between fresh feces and frozen feces in the treatment of *C. difficile* infection by FMT, frozen feces can only be stored for a limited period; moreover, feces cannot be promoted as easily and widely as drugs, which limits the clinical applications of FMT^{39,40}. 3) Before and after FMT, transplant recipients exhibit changes in intestinal bacterial diversity. Notably, the post-transplantation intestinal flora is not a simple combination of donor and receptor microbial communities; instead, it is a novel community of donor, receptor, and

environmental microbes. Donor microorganisms in receptor engraftment drive the mechanism of engraftment in a manner that is unclear. In a previous study, Petrof et al.⁴¹ isolated 33 types of bacteria from the feces of healthy people, which they used to culture a complex bacterial mixture. Two patients with recurrent *C. difficile* infection were successfully cured by FMT, using this mixture. This approach can effectively avoid the limitations of conventional FMT. Therefore, it is important to understand the effective and overall distributions of flora in feces, to facilitate standardization, mechanistic research, and promotion of FMT.

FMT began in ancient China, at least 1700 years ago.² It was first recorded by Ge Hong of the Eastern Jin dynasty, who wrote that “the huanglong decoction is best if it is aged for a long time. Drink excrement juice one liter, recover from illness immediately”. The processing technology of “fecal juice” and “huanglong decoction” was continuously updated. Subsequent generations of Chinese traditional medicine doctors made mature “gold juice,” which became a common medicine in the Ming and Qing dynasties. Therefore, based on this relationship between ancient “gold juice” and FMT, analysis of microbial components in gold juice may help to address some limitations of FMT.

On the basis of “*Tripterygium wilfordii* processing medicinal solution”,¹⁴ “Chinese Herbs”,¹⁵ “New herbal medicine”,¹⁶ “Compendium of Materia Medica”,¹⁷ and other processing methods in the literature, the “clear spring water, devoid of foul odor” gold juice can be obtained; we hypothesize that the entire production process comprises a simulated intestinal anaerobic environment with a new biological context, including some fecal bacteria that are carefully screened and cultivated. This environment includes no light, relative absence of oxygen, and cool temperature;

these conditions produce changes in biodiversity and community structure. In the present study, we found that the microflora of the filtrate obtained after preliminary processing of feces were similar to microflora within the intestinal tract. Subsequent comparison of first gold juice and filtrate revealed changes in fecal microecological conditions, demonstrating that the biodiversity and community structure in gold juice and filtrate differed after processing. Bacterial phenotypes in gold juice and filtrate both contained anaerobic bacteria, with some aerobic and facultative anaerobic bacteria. In addition, Gram-negative bacteria were predominant, whereas only a few Gram-positive bacteria were found. The proportions of potentially pathogenic bacteria were comparable between samples.

Consequently, we used statistical methods to identify differences between gold juice and filtrate. *Alistipes*, the Bacteroidales s24-7 group, the Lachnospiraceae NK4A136 group, *Allobaculum*, uncultured Eubacteriaceae, *Comamonas*, and uncultured hydrogenophilaceae were only detected in the gold juice. *Alistipes* is negatively correlated with the occurrences of cancer, autism, and depression.⁴²⁻⁴⁴ The Lachnospiraceae NK4A136 group is closely associated with inflammatory bowel diseases;⁴⁵ the abundance of the Lachnospiraceae_NK4A136_group is reduced in inflammatory bowel diseases.^{46,47} *Allobaculum* affects tryptophan metabolism and is important in prevention of inflammatory diseases in the central nervous system and intestinal environment.⁴⁸ Excessive use of antibiotics early in life has been shown to reduce the abundance of *Allobaculum* and enhance the risks of various diseases (e.g., asthma, type 2 diabetes, inflammatory bowel disease, and milk allergy).⁴⁹ *Comamonas* is involved in degradation of phenols, quinolines, and steroids, as well as metabolism of nitrogen.⁵⁰⁻⁵² In summary, the characteristic flora of gold

juice mainly consists of two components: flora that synthesize sugars and amino acids by degrading substances and probiotic flora. The following bacteria are only present in the filtrate: the phylum Firmicutes; the family Lachnospiraceae; the genera *Bacteroides*, *Prevotella* 9, *Blautia*, *Roseburia*, *Faecalibacterium*, *Subdoligranulum*, and *Dialister*; the species *Eubacterium coprostanoligenes*; and the ambiguous taxon Ruminococcaceae UCG 014. Both *Bacteroides* and *Prevotella* 9 belong to the Bacteroidetes phylum, which is an important component of the mammalian gastrointestinal flora.⁵³

Importantly, we investigated the microbial components in gold juice using a modified traditional Chinese method; this method may differ from the approach used in ancient medicine, as well as the approaches used in other fecal microbiota processing methods. For example, Ott et al.⁵⁴ used sterile-filtered stools from healthy donors for patients with symptomatic chronic-relapsing *C. difficile* infection; they showed that these methods effectively eliminated symptoms in patients, whereas fecal microbiota did not. In addition, Zhang et al.⁵⁵ reported that transplantation of washed microbiota may be safer and more easily controlled approach, compared with FMT prepared manually. The previous and ongoing studies (both preclinical and clinical) will serve to advance the use of FMT in clinical practice. Because a limited number of fecal samples was used in the current study, further investigations with more samples are needed to confirm the findings in our current study.

In conclusion, the present study showed that, after healthy children's feces were processed with a traditional Chinese medicine method, the resulting gold juice contained distinct microbial components, compared with the filtrate obtained during the gold juice preparation process. The findings of this study may provide useful

information regarding the effective bacterial components in fecal specimens for clinical implementation of FMT in treatment of patients with *C. difficile* infection.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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