

RESEARCH ARTICLE

BIOME-Preserve: A novel storage and transport medium for preserving anaerobic microbiota samples for culture recovery

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Abstract

Sequencing-based protocols for studying the human microbiome have unearthed a wealth of information about the relationship between the microbiome and human health. But these microbes cannot be leveraged as therapeutic targets without culture-based studies to phenotype species of interest and to establish culture collections for use in animal models. Traditional sample collection protocols are focused on preserving nucleic acids and metabolites and are largely inappropriate for preserving sensitive anaerobic bacteria for later culture recovery. Here we introduce a novel microbiome preservation kit (BIOME-Preserve) that facilitates recovery of anaerobic bacteria from human stool. Using a combination of culture recovery and shallow whole-genome shotgun sequencing, we characterized the anaerobes cultured from fresh human stool and from human stool held at room temperature in BIOME-Preserve for up to 120 hours. We recovered several species of interest to microbiome researchers, including *Bifidobacterium* spp., *Bacteroides* spp., *Blautia* spp., *Eubacterium halii* (now *Anaerobutyricum hallii*), *Akkermansia muciniphila*, and *Faecalibacterium prausnitzii*. We also demonstrated that freezing at -80°C did not adversely affect our ability to culture organisms from BIOME-Preserve, suggesting that it is appropriate both as a transport medium and as a medium for longer-term ultra-cold storage. Together, our results suggest BIOME-Preserve is practical for the collection, transport, and culture of anaerobic bacteria from human samples and can help enable researchers to better understand the link between the microbiome and human health and how to leverage that link through novel microbiome-based therapeutics.

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Introduction

The human microbiome plays a fundamental role in health and disease. Most bacteria are found in the gastrointestinal (GI) tract and are primarily anaerobic and facultatively anaerobic microorganisms. A large portion of the research and analysis of the microbiome to date is based around 16S rRNA gene sequencing [1] and more recently whole genome shotgun (WGS) metagenomics sequencing, of stool samples as a proxy for the human GI microbiome

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: H.L. is an employee of Anaerobe Systems; S.C. is the president of Anaerobe Systems. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

[2] Such protocols were originally developed due to limitations of culture-based techniques for capturing the full microbial diversity in a sample, with some claiming that up to 99% of known microbial diversity is unculturable [3, 4]. While this is true of certain environments, recent studies have shown that much of the bacterial diversity present in human stool can indeed be cultured. Lau *et al.* [5] demonstrated that the majority of bacterial taxa detected via 16S rRNA gene sequencing can be cultured via a combination of culture conditions while Ito *et al.* [6] reported successful growth of eight “unculturable” species using commercially available culture media. Similarly, Browne *et al.* isolated in pure culture 137 species from characterized and novel families, genera, and species [7].

An increasing number of research groups are thus turning to culture-based approaches to complement sequencing-based protocols. Organisms isolated and phenotyped in the laboratory can be used to develop and test animal models of disease or may comprise organism collections that can serve as the basis for further research or in the development of microbial-based treatments. To fully understand and leverage the potential beneficial effects of the human microbiome as a disease treatment or health-boosting strategy, it is necessary to characterize living organisms.

The collection and successful culture of organisms from samples such as human stool is challenging and is not fully supported by current sample collection and preservation techniques. Obligate anaerobic organisms contained in the samples are rapidly killed in the presence of oxygen. Furthermore, accumulation of toxic metabolic byproducts including short chain acids and alcohols can kill microorganisms in the sample if metabolism and growth continues after collection. The growth and proliferation of some fast-growing organisms in a sample can outcompete and prevent the recovery or isolation of other more fastidious and slow growing organisms [8, 9].

A fresh sample is considered the gold standard, as collecting samples in a laboratory or clinical setting followed by immediate processing can increase recovery of live organisms. However, this approach is often impractical, as it limits the population for sample collection to those in the immediate vicinity of the laboratory or clinic and with the ability to provide a sample at the time it is needed. Samples can also be frozen upon collection, which stops the metabolic process and preserves the organisms in stasis [10] but freezing organisms without a form of cryoprotectant may damage cell membranes, and samples are typically collected from the general population outside of the laboratory where appropriate freezing may not be feasible or convenient. Furthermore, shipment of frozen samples is more expensive and can be prohibitive.

These challenges can be alleviated through a sample collection device designed specifically to preserve anaerobic organisms present in a sample for culture recovery. While there have been several advances in preservation methods for DNA, RNA, and metabolites [11] which often involve agents that kill microorganisms, there are few options for preserving anaerobic bacteria in the microbiome for culture recovery. Cary Blair Medium has been used by the clinical microbiology field for decades and is a possible solution to protect organisms in microbiome samples during sample transport to the lab. However, it is a semi-solid medium appropriate for fecal swab samples, not for portions of whole stool, even if small. It is also not a viable solution for protocols unable to ensure sample processing or freezing shortly after collection, as organism viability quickly drops. And while a potential solution called GutAlive [12] was recently reported, it is not available to the research community at large. The field still lacks an appropriate microbiome sample collection kit specifically designed to preserve anaerobic organisms for culture recovery.

We have developed a microbiome preservation kit to address this unmet need. To accurately assess the ability of our kit, BIOME-Preserve to preserve microorganisms from stool, we

used a combination of culture and whole genome shotgun sequencing to compare our kit to the gold standard of fresh stool. We demonstrate the efficacy of BIOME-Preserve to preserve the viability of over 85% of bacteria cultured from human stool (including low abundance species) at room temperature for up to five days and after frozen storage. We suggest that BIOME-Preserve is a viable solution for microbiome research laboratories in both academia and industry to preserve anaerobic bacterial species in microbiome samples for culture-based phenotypic studies.

Materials and methods

Human subjects

Three adult (18+ years of age) human donors volunteered and consented to provide a single whole stool sample for this study. The donors were asked to provide a sample using a commode specimen collection bucket (02-544-208, Fisher Scientific, Hampton, NH) and to bring the sample to the Anaerobe Systems laboratory as soon as possible after collection. The samples were immediately processed anaerobically upon arrival. Donors included one adult female in her 30s, one adult male in his 70s, and one adult male in his 40s with a verbally self-reported prior recurrent *C. difficile* infection. All donors self-reported verbally no active gastrointestinal infections or recent antibiotic usage at the time of sample collection. No identifying information were stored except for the donors' names and signatures on the consent forms. All experimental procedures were approved by Aspire IRB (Santee, CA; protocol # Anaerobe01).

Stool preprocessing

The donor stool sample was weighed and then moved into an anaerobic chamber (AS-580, Anaerobe Systems, Morgan Hill, CA) containing a gas mixture of 4.6% CO₂ / 5% H₂ / 90.4% N₂ as soon as possible after collection (within 30 minutes for donor 1, 60 minutes for donor 3, and 4 hours for donor 2). Inside the anaerobic chamber, the stool was transferred to a Ninja 400-watt blender (QB900B Shark Ninja, Needham, MA). Chilled (4°C) and pre-reduced anaerobically sterilized (PRAS) dilution blank medium (AS-9183, Anaerobe Systems) was added at a volume of 2:1 (2mL per gram) of the stool weight. The sample was blended until fully homogenized (approximately 4 minutes). A 0.5mL aliquot of homogenized stool was collected into a 2mL cryovial (#431386, Corning Life Sciences, Corning, NY) and frozen at -80°C until processing for WGS sequencing and analysis.

While in the real world, stool will most likely not be homogenized inside an anaerobic chamber, we chose first exposure to oxygen to be upon inoculation into BIOME-Preserve for two reasons: 1) we tested the effects of the ingredients of BIOME-Preserve on bacterial viability in general using a T0 tube inoculated inside the anaerobic chamber (see below for more details), and therefore needed to eliminate the confounding effect of oxygen introduced during homogenization, and 2) most real world situations do not actually homogenize whole stool prior to inoculation into transport or other medium; instead, a piece of whole stool is collected (or swabbed) and moved into the desired container in the field or laboratory. Because several studies have demonstrated “regional” differences in bacterial composition along stool samples, we wanted to test the performance of BIOME-Preserve when using gold standard stool (i.e., fresh homogenized) to obtain an accurate assessment of its ability to preserve organisms alive. Killing some organisms during homogenization, if done outside of the chamber, would have prevented us from knowing exactly how many organisms the product can preserve. Additionally, homogenization ensured consistency across replicates as the same exact community at the same proportions would have been inoculated into every BIOME-Preserve tube. After

inoculation of the T₀ BIOME-Preserve tube, homogenized stool was removed from the anaerobic chamber for inoculating the remaining BIOME-Preserve tubes in air as described below.

A sequential dilution of homogenized stool into PRAS Yeast Casitone Fatty Acid with Carbohydrate (YCFAC) broth (AS-680, Anaerobe Systems) was performed to obtain a 10⁻⁵ dilution, which was used for high density growth and in serial dilutions for low density growth on solid media as described below.

Assessment of BIOME-Preserve microbial toxicity

BIOME-Preserve is a proprietary anaerobic transport medium device designed specifically to preserve anaerobic organisms in whole microbiome samples for later culture recovery. The product has a provisional international patent (application number PCT/US2020/036457; published October 12, 2020 under publication number WO/2020/247846).

A T₀ BIOME-Preserve control tube (see Fig 1) was inoculated after homogenizing fresh stool to assess inhibitory effects of the components contained in the BIOME-Preserve medium on the viability of organisms cultured from stool. Homogenized stool (1.0 mL) was added directly to a single BIOME-Preserve tube (9 mL) then held at room temperature for 1 hour. Inoculation and holding were performed inside of an anaerobic chamber to eliminate any confounding effects due to oxygen.

After one hour, a sequential dilution of the T₀ BIOME-Preserve tube into YCFAC broth was performed. The 10⁻⁵ dilution was then used for high-density and low-density culturing on solid media as described below. A single replicate set of media were inoculated for the T₀ BIOME-Preserve control sample as follows: 100uL of the 10⁻⁵ dilution were streaked onto PRAS Brucella Blood Agar (BRU, AS-141, Anaerobe Systems) and PRAS Yeast Casitone Fatty Acid with Carbohydrate and Blood (YCFAC-B, AS-677) agar plates.

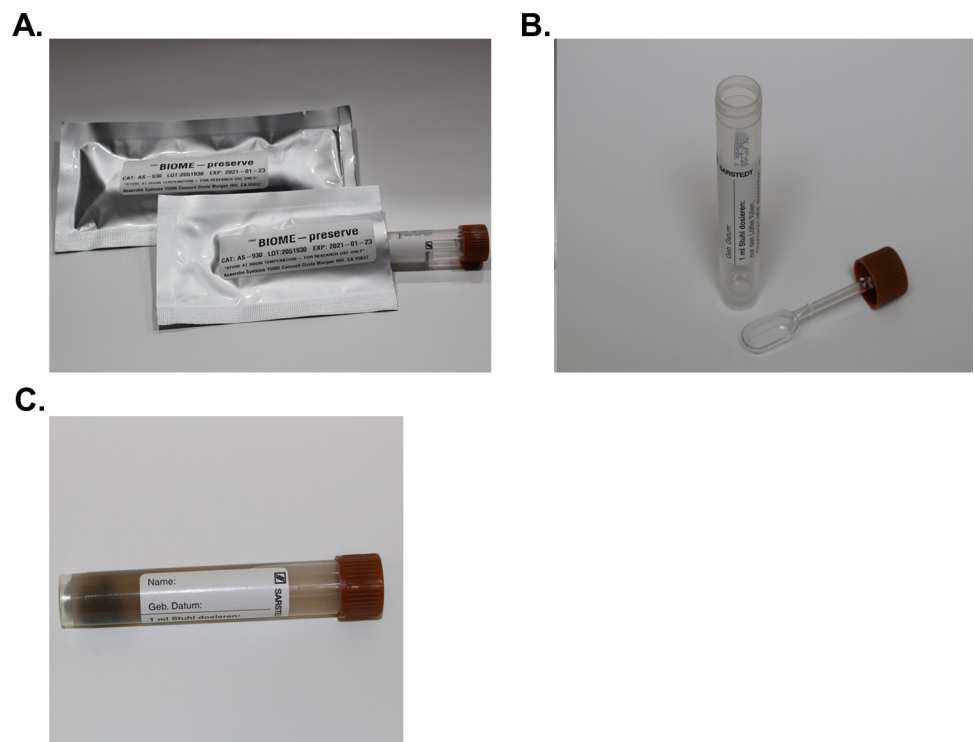


Fig 1. BIOME-Preserve tube.

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BIOME-Preserve tube inoculation

To test the ability of BIOME-Preserve to preserve anaerobic bacteria from fresh human stool, homogenized stool (1.0 mL) was added directly to two replicate BIOME-Preserve tubes (9.0 mL) and held at room temperature for 72 hours or 120 hours. These time points were chosen based on results from prior experimental data from a single donor showing minimal loss in successfully cultured bacteria between 72 and 120 hours (data publicly available from ENA under accession number PRJEB45653) and also to encompass the most likely scenarios for sample collection and/or mail transit time.

The tubes were inoculated outside of the anaerobic chamber to mimic traditional sample collection in air. At the end of each specified holding period, the tubes were inverted to mix contents, and a 0.5mL aliquot was collected from each tube and stored at -80°C . Then, 217 μL were transferred from each tube to each of two replicate 7mL YCFAC broth tubes to create a 10^{-3} YCFAC BIOME-Preserve dilution. The 10^{-3} dilution was then used for high density growth and in serial dilutions for low density growth on solid media as described below.

CFU determination for fresh stool and BIOME-Preserve tubes

To determine the CFU of fresh stool and for inoculating low-density growth plates, we performed serial dilution, targeting 10^{-6} – 10^{-8} dilutions. This process was performed twice to create two serial dilution replicate sets. The 10^{-6} – 10^{-8} dilutions were inoculated (100 μL) onto PRAS BRU solid medium plates and PRAS YCFAC-B solid medium plates in triplicate. The plates were incubated in the anaerobic chamber at 37°C for 120 hours. Photographs were taken of each plate, and colonies were counted for the plates in the countable range of 30–300 colonies (see [S1 Fig](#) for examples of CFU and high-density culture plates). CFUs from BIOME-Preserve tubes were assessed following the same protocol, except 10^{-4} – 10^{-6} dilutions were plated.

Bacterial colonies were carefully removed from plates with approximately 2,000 isolated colonies using the beveled edge of a cell lifter (08-100-240, Fisher Scientific, Hampton, NH) and transferred to a 2mL cryovial. Harvested cells from the three replicate plates from a single dilution replicate were combined into a single cryovial. 2mL of dilution blank were added to the cryovials, the cellular material was mixed via mechanical mixing, pipet mixing, and repeated inversion of the tube, and 1mL of material was transferred to a new 2mL cryovial. All cryovials were frozen immediately at -80°C ; one of each duplicate tube was sent for WGS sequencing and analysis.

High density culture of microbes from fresh stool and BIOME-Preserve tubes

To determine which microbes could be recovered from fresh stool using varying media types, 100 μL of the 10^{-5} YCFAC stool dilution were streaked onto triplicate plates of the following solid media: YCFAC-B, BRU, and PRAS Chocolate Agar (CHOC, AS-244, Anaerobe Systems). Two replicate sets of media were inoculated. The plates were incubated anaerobically for 120 hours at 37°C . Following the procedure for the CFU plates described above, bacterial mat material was removed from the plates, transferred to 2mL cryovials, and split into two cryovials for a final suspended volume of 1mL. All cryovials were frozen immediately at -80°C ; one of each duplicate cryovial was sent for WGS sequencing and analysis. Contents of BIOME-Preserve tubes were streaked for high-density culture following the same protocol. In a pilot study, we observed a 2-fold reduction in CFU from BIOME-Preserve compared to fresh stool (see [S2 Fig](#) for example photos taken for CFU counting). Therefore, to ensure comparable cell

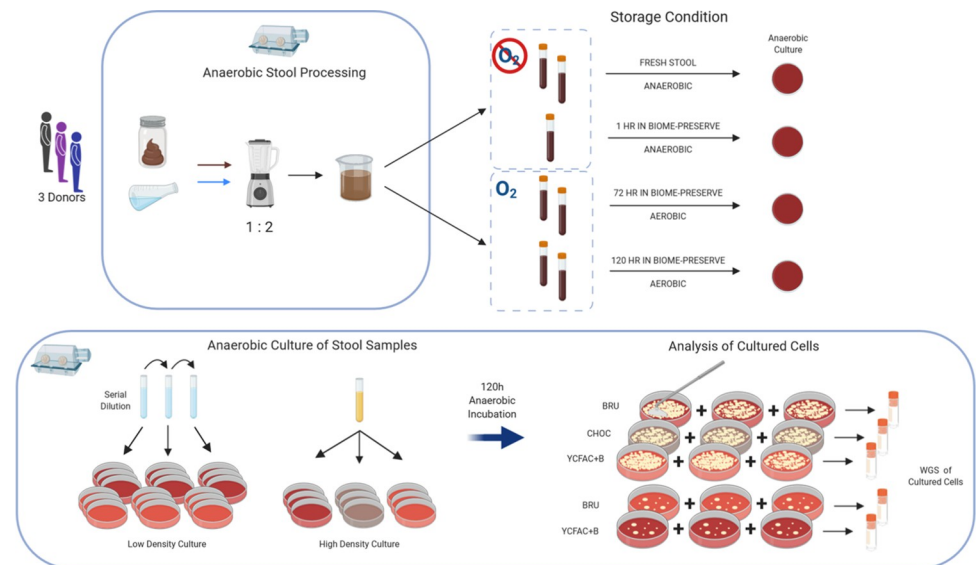


Fig 2. Experimental process. Schematic of stool collection, BIOME-Preserve inoculation, and culture of homogenized stool and BIOME-Preserve tube contents for subsequent WGS sequencing and analysis for species identification. This process was repeated 3 times for each of the 3 stool donors.

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numbers were inoculated onto all solid media plates in the current study, we plated the 10^{-3} YCFAC BIOME-Preserve dilution and not the 10^{-5} dilution as was the case with fresh stool. We confirmed this through the CFU counts from solid medium plates inoculated with fresh stool and BIOME-Preserve tube contents. A full schematic of sample collection and processing is illustrated in Fig 2.

Shotgun sequencing and data analysis

To identify successfully cultured species, all cryovials collected as outlined above were sent to CoreBiome (St. Paul, Minnesota, USA) for DNA extraction and shallow WGS sequencing and analysis (BoosterShot). BoosterShot yields an average of 2 million reads per sample and enables species- and strain-level taxonomic classifications. Our samples were sequenced on an Illumina NovaSeq 6000 sequencer with a 2 x 150 flow cell. The average number of reads per sample was 2,898,954.882; the maximum number of reads was 8,014,666 and the minimum number of reads was 388,341. A full sequence report illustrating total number of reads as well as number reads mapped to genus, species, and strains, per sample, can be seen in [S1 Table](#).

Additionally, six samples from donor 1 were also selected for deep metagenomic sequencing (DeepSeq), also performed by CoreBiome. As described in more detail below, these results were used to validate the minimum relative abundance of a species, according to BoosterShot, to consider a species successfully cultured. The average read depth achieved for our six DeepSeq samples was 187,462,994.2, with a maximum of 357,652,562 and a minimum of 111,694,884. A full sequence report illustrating total number of reads as well as number reads mapped to genus, species, and strains, per sample, can be seen in [S2 Table](#).

Host DNA was removed from the sequencing data prior to delivery to Anaerobe Systems and then aligned, assigned taxonomy, and grouped into OTUs (where each OTU is a unique species) as described in [13]. Briefly, DNA sequences were aligned to a curated database containing all representative genomes in RefSeq for bacteria with additional manually curated strains. Reads were mapped at 97% alignment identity to the CoreBiome Venti database

(contains whole-genome sequences of 19,840 strains, up to 20 strains per species) using the BURST aligner [14] and assigned taxonomies based on those alignments. Reads mapping to more than one genome were assigned taxonomies using the lowest common ancestor that was consistent across at least 80% of all reference sequences tied for best hit.

OTU counts for each OTU were normalized to the OTU's genome length and then any OTUs accounting for less than one-millionth of all species-level markers across the entire data set were removed. Additionally, any OTUs with less than 0.01% of their unique genome regions and less than 1% of their total genome covered were removed. A rarefied OTU table (10,000 reads/sample) was used for downstream analyses (S3 Table). The same filtering process was applied to both BoosterShot and DeepSeq sequencing data.

To determine which species we successfully cultured using our methods, we considered only OTUs with a taxonomic classification at the species level (i.e. unknown species with genus, family, or higher classifications were not considered). Because sequencing results can also reflect DNA from dead organisms, it was important to be confident that the species we identified on our culture plates represented bacteria that had actually grown on the plates rather than simply DNA contained in the inoculum. Therefore, we used the DeepSeq data to validate the relative abundance cutoff in BoosterShot data for considering a species successfully cultured.

Using CoreBiome's coverage cutoffs for including an OTU in the OTU table as a guide (1% for whole genome coverage and 0.01% for unique region coverage) and comparing these DeepSeq data to the shallower BoosterShot sequencing data, we considered a species successfully cultured if its average relative abundance according to BoosterShot data, across all replicate media plates, was 0.01%.

To determine the change in relative abundance of cultured species between stool and BIOME-Preserve tubes and across time in BIOME-Preserve, we first determined which species were successfully cultured from fresh stool, and then compared each species' relative abundance in fresh stool culture to its relative abundance in BIOME-Preserve culture to determine the percentage drop in relative abundance. Results were stratified according to fold-change in relative abundance (less than 1-fold, 1-fold, or 2-fold +).

Sequences and metadata are available from the European Nucleotide Database under accession number PRJEB40933.

Results

In this study, we cultured anaerobic bacteria from fresh stool as well as stool inoculated and held in BIOME-Preserve for 72 hours and for 120 hours from three different human donors. We used three different culture media to capture a range of bacterial diversity, from highly abundant species to rarer, slow growing and/or fastidious microorganisms. Using serial dilutions, we plated high density and low-density growth culture plates, with low density culture plates used to target slow growing species that are easily outcompeted. Shallow whole genome shotgun sequencing was used to identify species that were successfully cultured, and which were impacted by storage in BIOME-Preserve.

Bacteria cultured from fresh stool

Because of the inherent variability between individual people, we collected samples from three different adult donors, male and female, from 30–70 years of age. Although all donors were healthy (self-reported) at the time of sample collection, one donor had experienced a recurrent *Clostridioides difficile* infection a few years prior to the study, and verbally reported continued gastrointestinal issues believed by the donor to be related to the previous infection. No medical records were accessed or used to confirm any donor's health status.

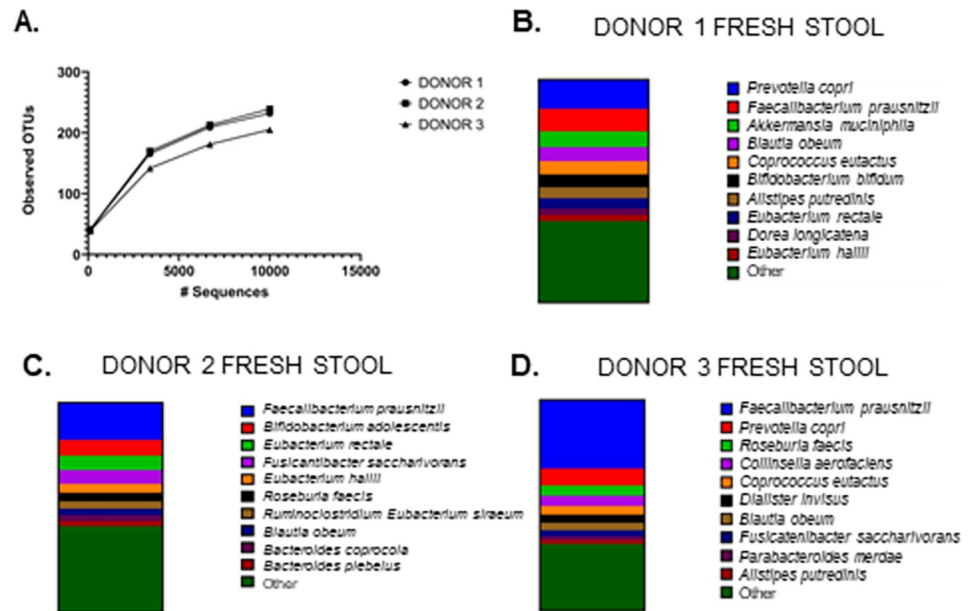


Fig 3. Donor-specific bacterial communities. Shallow WGS sequencing of homogenized stool contents reveals a variety of bacterial species present across the three human donors in this study. (A) Rarefaction curve illustrating total number of observed OTUs per donor; samples rarefied to 10,000 sequences per sample max. The top ten species detected in donor 1 (B), donor 2 (C), and donor 3 (D), are plotted, with remaining species grouped into “other”.

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As described in the methods section above, we utilized DeepSeq data to validate our chosen relative abundance cut-off from BoosterShot data for identifying anaerobes recovered on culture media. We assessed the percent whole genome and unique genomic regions coverage, per DeepSeq, of all species with a relative abundance of 0.01%, per BoosterShot, in culture material collected from media plates. As S4–S6 Tables illustrate, the genomes of at least one strain per each of these species were sufficiently covered to consider those species successfully cultured.

Comparing sequencing data only, we observed three unique bacterial communities in terms of diversity and composition (Fig 3). All three donors were dominated by species in the Firmicutes phylum (53.5% for donor 1, 62.7% for donor 2, and 67.2% for donor 3, see S3 Fig), while all had a similar relative abundances of Bacteroidetes. Proteobacteria were detected at 2.3% and 1.3% relative abundance in donor 2 and donor 3, respectively, but less than 1% relative abundance in donor 1. Notably, Verrucomicrobia were detected only in donor 1. The diversity of species present (see S7–S9 Tables) across the donors provided a good foundation to test the ability of BIOME-Preserve to preserve a variety of bacterial species across different individual donors.

Using three different culture media and two dilution factors, we successfully cultured, from fresh stool, 173 species from donor 1, 168 species from donor 2, and 168 species from donor 3. Importantly, several of these species are of interest to microbiome researchers, including several *Bifidobacterium* and *Bacteroides* species, *Blautia* species, *Akkermansia muciniphila*, and *Faecalibacterium prausnitzii*. Species cultured from fresh stool represented our control against which species cultured from BIOME-Preserve were compared.

Assessment of the effect of BIOME-Preserve contents on stool microorganisms

Before determining which species could be cultured from stool held in BIOME-Preserve, we first determined whether the additives in BIOME-Preserve harmed anaerobic organisms from

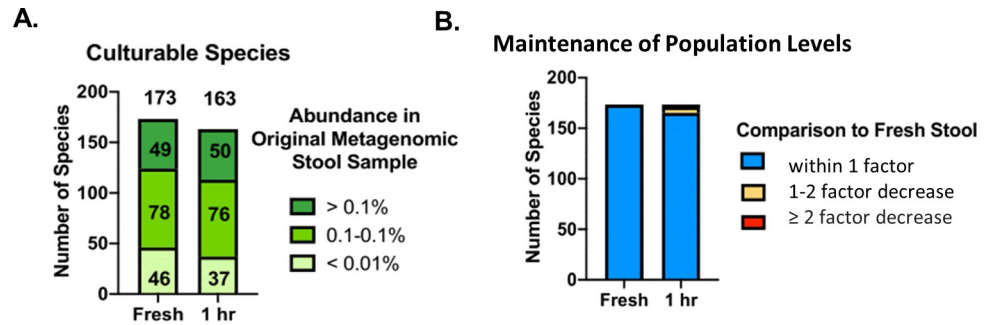


Fig 4. Effect of BIOME-Preserve on anaerobe viability. A) The number of species recovered from fresh stool and the T₀ BIOME-Preserve tube, stratified by relative abundance in fresh stool. B) The relative abundance of species cultured both from fresh stool and from the BIOME-Preserve T₀ tube, stratified by change in relative abundance. The majority of cultured species had less than a 1-fold drop in relative abundance.

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human stool. We sequenced cultured cells from homogenized stool collected from donor 1 and from stool held in BIOME-Preserve for 1 hour. It is important to note that the 1-hour tube, unlike the other BIOME-Preserve tubes, was inoculated and held under anaerobic conditions to eliminate any harmful effects of temporary exposure to oxygen and ensure the only variables tested were the BIOME-Preserve additives.

As seen in Fig 4, there was minimal difference in the total number of cultured species from the BIOME-Preserve tubes held for 1 hour compared to fresh homogenized stool. A total of 163 species were recovered on culture media from the T₀ BIOME-Preserve tube, compared to 173 species recovered from fresh, homogenized stool. A similar proportion of high, middle, and low-abundance species in fresh stool (as determined via WGS) were recovered from fresh stool culture and T₀ BIOME-Preserve culture.

Effects of holding time in BIOME-Preserve on cultured anaerobes

We next determined whether inoculation outside of the anaerobic chamber (i.e., in the regular laboratory environment) and longer holding times had any effect on the anaerobes that could be cultured from BIOME-Preserve compared to anaerobes that could be cultured from fresh stool. We held BIOME-Preserve tubes inoculated with homogenized stool at room temperature for two time periods (72 hours and 120 hours), chosen for relevance to typical lengths of time it takes for samples to arrive in the laboratory from the field, or to transit from one location to another for processing.

Sequencing of culture material collected from solid media revealed that we cultured 163 and 183 species from donor 1 samples held in BIOME-Preserve for 72 and 120 hours, respectively, 145 and 133 from donor 2, and 144 and 139 from donor 3 (Fig 5A). We recovered from BIOME-Preserve species at both high and very low abundances in fresh stool (Fig 5B), as well as a small number of species that we did not recover from fresh stool.

Considering only those species that were cultured both from fresh stool and from BIOME-Preserve samples, we cultured 88% of species cultured from stool from BIOME-Preserve after 72 hours and 87% after 120 hours for donor 1. Those numbers were 83% and 77% for donor 2, and 89% and 81% for donor 3 (Fig 5C). Across all donors, we cultured an average of 87% of species cultured from stool after 72 hours in BIOME-Preserve, and 82% after 120 hours.

As expected, we observed medium-dependent recovery of bacterial species, with CHOC and BRU agar yielding similar microbial communities (Fig 6). As seen in S10 and S11 Tables, YCFAC-B agar performed the poorest in terms of the number of species it was able to recover, but it was able to recover specific organisms that CHOC and BRU agar could not. These results

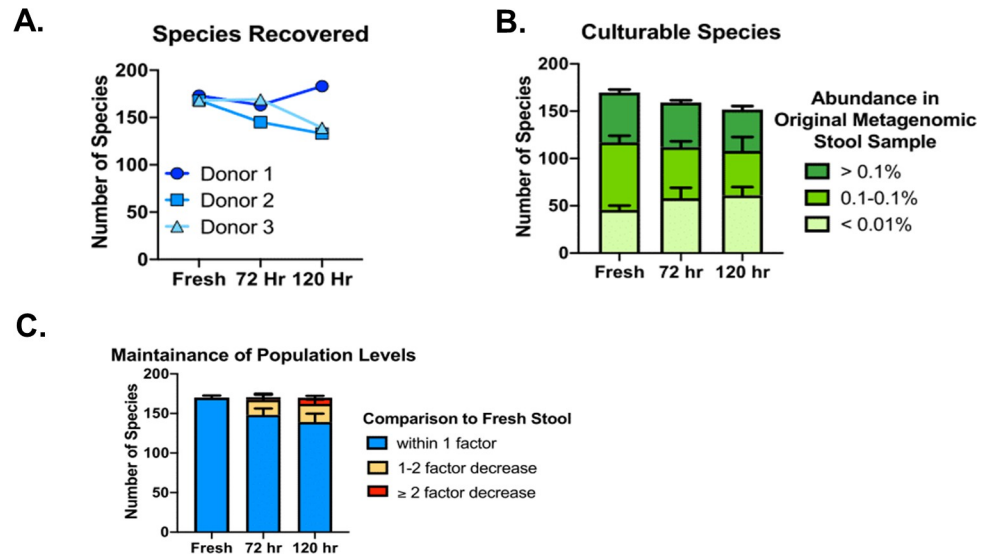


Fig 5. Ability of BIOME-Preserve to maintain anaerobe viability over time. A) The number of species recovered from fresh human stool and from BIOME-Preserve after 72 and 120 hours. B) Species cultured from BIOME-Preserve tubes stratified by relative abundance in fresh stool. C) Species cultured from stool that were also cultured from BIOME-Preserve tubes after 72 hours and 120 hours, stratified by fold-change in relative abundance. The majority of cultured species had less than a 1-fold drop in relative abundance.

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support the need for multiple media types to recover the majority of bacterial diversity present in human stool.

Because we plated low-density plates in addition to high density plates to increase our chances of culturing easily outcompeted slow-growing organisms and lower abundance species, we next assessed which organisms were more easily cultured on low-density plates. Several species had a greater than 100% increase in relative abundance on low-density plates compared to the high-density plates of the same media type. As seen in Table 1, most of these organisms fit into seven genera: *Bacteroides*, *Collinsella*, *Lachnoclostridium clostridium*, *Roseburia*, and *Streptococcus*, although several species belonging to other genera were also captured, including *Akkermansia muciniphila*.

Effects of ultra-cold storage on BIOME-Preserve

Because many microbiome studies do not process samples immediately but store them frozen for some time prior to processing, we previously compared the growth of anaerobes cultured from BIOME-Preserve held at room temperature to the growth of anaerobes cultured from BIOME-Preserve held at room temperature and then frozen at -80°C for one week. Using a stool sample provided by donor 1, we homogenized the stool sample and inoculated BIOME-Preserve tubes as described for the current three-donor experiment in the materials and methods section, and additionally moved a subset of BIOME-Preserve tubes into a -80°C freezer for 1 week prior to culture recovery. Culture material was collected and sequenced using the BoosterShot protocol, and all samples and sequences can be accessed from ENA under accession number PRJEB45653. Of note, this was a preliminary study performed prior to product patent and therefore the naming convention “MACM” is seen on sample metadata rather than “BIOME-Preserve.”

Comparing culture recovery from unfrozen BIOME-Preserve and frozen BIOME-Preserve, we observed that 90% of the anaerobes cultured from unfrozen BIOME-Preserve were also

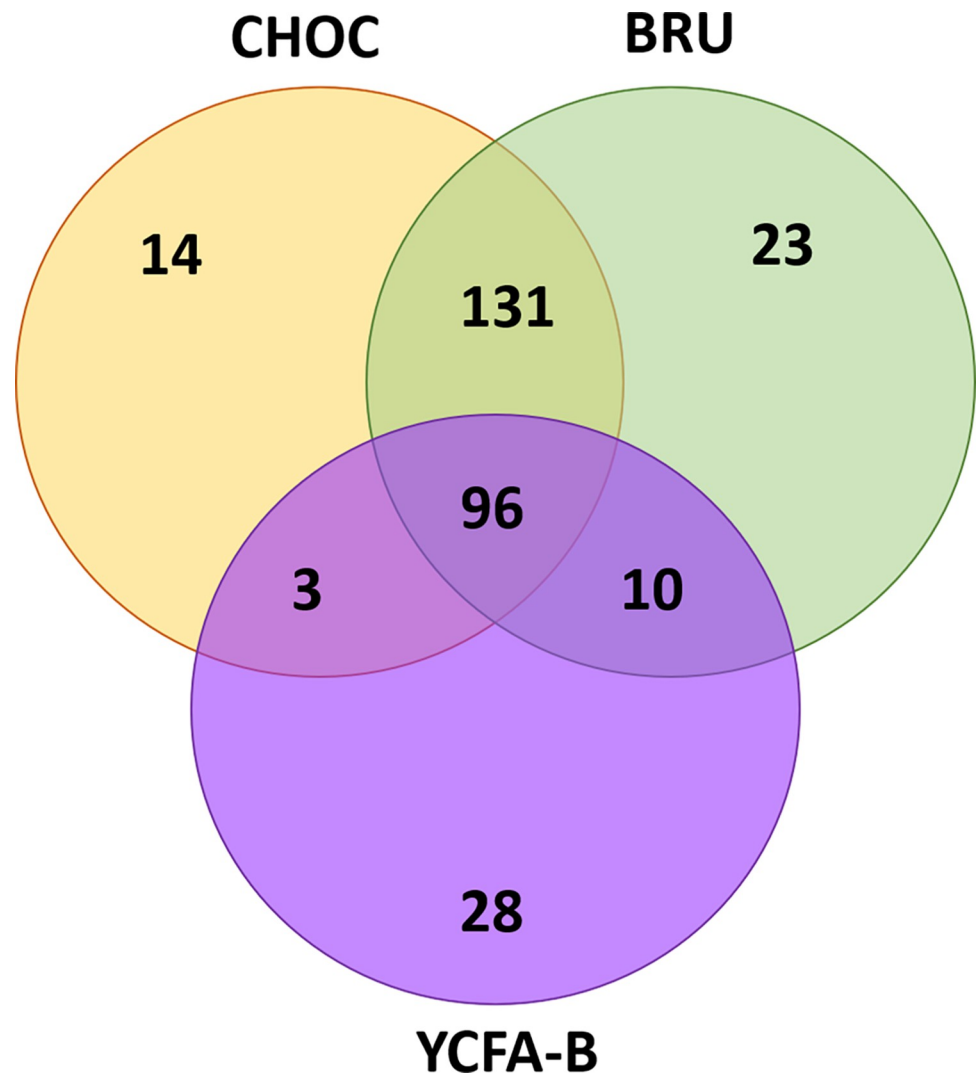


Fig 6. Medium-dependent recovery of anaerobes from BIOME-Preserve. Venn diagram illustrating media-specific growth of anaerobic bacteria. CHOC and BRU solid media captured many of the same species; YCFAC-B solid medium facilitated growth of a different set of bacterial species.

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cultured from BIOME-Preserve stored at -80°C (Table 2). These results suggest that a single freeze-thaw cycle did not hinder our ability to culture organisms at very low abundance in fresh stool from the BIOME-Preserve samples. We also saw similar recovery statistics in this preliminary study as in the current three-donor study, with over 80% of species cultured from fresh stool also cultured from BIOME-Preserve samples. All data comparing the relative abundances of species detected on culture media across all samples (fresh stool, BIOME-Preserve, and frozen BIOME-Preserve) can be seen in S12–S14 Tables, and the original, rarefied OTU table is presented as S15 Table.

Discussion

Here we demonstrate that BIOME-Preserve is an effective method for preserving anaerobic bacteria for culture recovery for up to 5 days. From stool samples of 3 donors and using three media types and two dilution factors, we cultured from fresh stool an average of 170 species.

Table 1. Change in relative abundance of anaerobes from high and low dilutions.

	Average Change Across All Donors	D1 Change, Stool	D2 Change, Stool	D3 Change, Stool
<i>Akkermansia muciniphila</i>	81%	na	0%	162%
<i>Bacteroides barnesiae</i>	192%	na	192%	na
<i>Bacteroides caccae</i>	455%	na	455%	na
<i>Bacteroides cellulosilyticus</i>	152%	na	225%	79%
<i>Bacteroides egerthii</i>	178%	na	283%	74%
<i>Bacteroides fingoldii</i>	56%	27%	153%	-13%
<i>Bacteroides intestinalis</i>	245%	278%	213%	na
<i>Bacteroides oleiciplenus</i>	151%	na	151%	na
<i>Bacteroides plebeius</i>	68%	na	123%	14%
<i>Bacteroides</i> sp. 2 1 33B	130%	117%	144%	na
<i>Bacteroides</i> sp. 2 2 4	160%	na	160%	na
<i>Bacteroides</i> sp. 3 1 13	366%	na	366%	na
<i>Bacteroides</i> sp. Marseille-P3208T	297%	na	297%	na
<i>Bacteroides xylanisolvens</i>	47%	na	119%	-25%
<i>Collinsella intestinalis</i>	171%	na	261%	80%
<i>Lachnospirillum Clostridium lavalense</i>	956%	796%	1902%	170%
<i>Lachnospirillum Clostridium symbiosum</i>	102%	na	102%	na
<i>Roseburia faecis</i>	1525%	na	1525%	na
<i>Roseburia hominis</i>	113%	13%	213%	na
<i>Roseburia intestinalis</i>	113%	-12%	373%	-21%
<i>Streptococcus anginosus</i>	2552%	2552%	na	na
<i>Streptococcus australis</i>	290%	290%	na	na
<i>Streptococcus oralis</i>	1642%	na	na	1642%
<i>Streptococcus parasanguinis</i>	114%	114%	na	na
<i>Streptococcus</i> sp. 1171 SSPC	951%	951%	na	na
<i>Streptococcus</i> sp. 400 SSPC	911%	911%	na	na
<i>Streptococcus</i> sp. A12	885%	885%	na	na
<i>Streptococcus</i> sp. F0441	21446%	na	na	21446%
<i>Streptococcus</i> sp. HMSC034B04	1287%	1287%	na	na
<i>Streptococcus</i> sp. HMSC034F02	2773%	2773%	na	na
<i>Streptococcus</i> sp. HMSC056D07	3484%	3484%	na	na
<i>Streptococcus</i> sp. HMSC062D07	224%	224%	na	na
<i>Streptococcus</i> sp. HMSC073A12	1538%	1538%	na	na
<i>Streptococcus</i> sp. I-G2	547%	547%	na	na
<i>Streptococcus</i> sp. I-P16	523%	523%	na	na
<i>Streptococcus</i> sp. oral taxon 071	9512%	na	na	9512%
<i>Streptococcus urinalis</i>	2601%	2601%	na	na

Percent change in relative abundances of species more successfully recovered on low density plates compared to high density plates (species recovered from fresh stool). D1 = donor 1, D2 = donor 2, D3 = donor 3.

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After 72 hours in BIOME-Preserve, we cultured an average of 150 species, and after 120 hours in BIOME-Preserve we also cultured an average of 150 species; at both time points we cultured a small number of species that were not recovered from fresh stool.

Of the species cultured from fresh stool and BIOME-Preserve samples, 87% were minimally impacted by 72 hours in BIOME-Preserve; this number dropped to 82% after 120 hours. Because the BIOME-Preserve tubes were inoculated, capped, and held outside of an anaerobic chamber, these numbers are reflective of expected results given typical laboratory procedures.

Table 2. Anaerobes cultured from stool, BIOME-Preserve, and frozen BIOME-Preserve.

Fresh Stool	72 hr BIOME-P (% of fresh stool)	72 hr + freeze-thaw BIOME-P	120 hr BIOME-P (% of fresh stool)	120 hr + freeze-thaw BIOME-P
		(% of fresh stool)		(% of fresh stool)
187	177 (94.6%)	174 (93%)	164 (87.7%)	151 (80.7%)

Number of species successfully cultured from fresh stool, BIOME-Preserve held at room temperature, and BIOME-Preserve held at room temperature and then frozen at -80°C for one week.

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Further protection may be afforded if stool is added to BIOME-Preserve inside of an anaerobic chamber. Because such an approach is not always feasible, especially during field studies, we only assessed the efficacy of BIOME-Preserve in the most likely real-world scenario.

The change in relative abundance of anaerobes between fresh stool and BIOME-Preserve was within 1-fold for the vast majority of anaerobic species that we cultured from both, with a very small number experiencing a 2-fold change in relative abundance. Considering the average human gut contains $\sim 10^{13}$ microorganisms [15], a 2-fold change in relative abundance still equates to large numbers of bacterial cells and demonstrates that BIOME-Preserve is much more stringent for maintaining live organisms than any other available method. For example, we cultured *Faecalibacterium prausnitzii* from both stool and BIOME-Preserve with a 1-fold change in relative abundance in BIOME-Preserve; by comparison, Papanicolas *et al.* [16] reported a 12-fold loss in this species when stool samples were processed aerobically vs. anoxically. This suggests that BIOME-Preserve could be an appropriate tool for preserving microbiome communities intended for therapeutic purposes such as FMT or for other downstream applications such as studies using humanized animal models. It can also substantially reduce the laboratory resources needed at collection sites.

We collected stool from three adult humans, two male and one female, one of which was elderly and one of which had a self-reported previous recurrent *C. difficile* infection, to assess the ability of BIOME-Preserve to protect a wide variety of organisms from different human individuals. While the microbial communities were notably different from one another, there were no donor-specific effects on our ability to recover organisms from fresh stool or from BIOME-Preserve. We cultured 173 species from donor 1, 168 species from donor 2, and 168 species from donor 3 with 85%, 85%, and 80% of those species experiencing a less than 1-fold difference in relative abundance after 5 days in BIOME-Preserve. With 381, 516, and 426 species detected via WGS sequencing in donor 1, 2, and 3, respectively, our protocol successfully cultured, on average, 39% of fecal species detected via sequencing. Notably, a single freeze-thaw cycle had minimal effect on the anaerobic bacteria we successfully cultured from BIOME-Preserve.

Our results, based strictly on the number of species recovered, are similar to those reported by Browne, *et al.*, [7] who successfully cultured 137 species, including 45 candidate novel species using only a single medium type (YCFA). Based on percentages of what is detected in stool, we recovered 20% less than that reported by Ito *et al.* (61%), and Lau *et al.* [5] reported culture success rates over 90%. Those two groups used 27 and 33 different media, respectively, and Lau *et al.* used anoxic and aerobic culture conditions (5); the vast number of plates utilized by these two protocols provides many more chances to obtain very low abundance species and easily outcompeted species compared to our protocol. Cultures consisted of a vast and highly diverse population with many factors such as sampling variation, plate spreading, and inter-community growth all affecting whether or not a species could be recovered on culture media. We also did not count every species with a relative abundance $>0\%$ on culture media as successfully cultured; therefore, based on our cutoff (at least 0.01% relative abundance), some species that may have indeed grown on culture are not included in our percentages.

Additionally, it is difficult to perform direct comparisons; for example, Browne *et al.* recovered less species than we in terms of sheer number but outperformed in terms of percentage compared to stool (72%). Such variability could be due to cross-donor differences as the number of species present in a “typical” human gut can range from 500–1,000 [17, 18]. Additionally, the number of species detected via sequencing may not be representative of the truly culturable proportion, as some DNA could belong to transient organisms no longer present or to dead organisms and therefore may just be artifacts. Sequencing method and taxonomy databases can also affect results; we used WGS sequencing and analysis, which provides more accurate species calls than 16S rRNA gene sequencing; however, we also eliminated any OTUs that did not receive a confident species-level identification. While these OTUs do represent individual species, we found it more valuable to the community to provide information on known species at this time; had we included these OTUs in our final counts our final numbers may have been more comparable to those achieved by other groups.

Based on results from other groups, we chose three media types to maximize the bacterial diversity we were able to culture: Brucella blood agar, Chocolate agar, and Yeast Casitone Fatty Acid agar supplemented with carbohydrates and blood. The most abundant species present in all three donors according to WGS sequencing were easily cultured using these three media types. We also successfully cultured several anaerobes that were at very low abundance in the original stool samples, as well as several species of interest to the microbiome research community. Notably, two species of interest (*Prevotella copri* and *Faecalibacterium prausnitzii*) were recovered at a higher relative abundance on low density plates than on high density plates, suggesting that more dilute samples may be necessary to capture slow growers and easily outcompeted organisms. Indeed, other studies have reported difficulty supporting *Prevotella* spp. viability in other transport media [19, 20]; our ability to recover it, albeit at lower rates than other microorganisms, suggests that BIOME-Preserve could be an appropriate tool for recovering these species given appropriate media type(s) and dilutions used for inoculation onto solid media.

Brucella blood agar and Chocolate agar yielded similar microbial communities on culture. Both successfully recovered several members of the *Bacteroides* genus that were not well-recovered using YCFAC-B agar. This includes *Bacteroides fragilis*, which has recently been dubbed a “next-generation” probiotic [21] and has been tested in the context of several disease states, including autism spectrum disorder [22]. On the other hand, YCFAC-B was particularly useful compared to BRU and CHOC agar for recovering a wide range of common human anaerobes, including the butyrate producers *Eubacterium rectale* and *Faecalibacterium prausnitzii*, a major component of the healthy human microbiome and an important contributor to intestinal health [23–25]. YCFAC-B also enabled recovery of several low-abundance (<0.01%) *Streptococcus* and *Lactobacillus* species, both from fresh stool and from BIOME-Preserve samples. All three media types recovered several *Bifidobacterium* and *Blautia* species; some *Blautia* recovered better on YCFAC-B while others did better on BRU or CHOC. *Blautia* is of increasing interest to the research community due to its inverse association with obesity, visceral fat, disturbances in glucose metabolism, and intestinal inflammation [26, 27].

Of note, while we successfully cultured several *Prevotella* species from fresh stool on all three media types, we struggled to culture one species of great interest to the microbiome research community, *Prevotella copri*, from BIOME-Preserve samples, only capturing it at one time point (72 hour BIOME-Preserve) from a single donor (donor 3) and at very low relative abundance (0.07%). The current experimental methods were not sufficient to determine confidently whether these results are due to toxicity from BIOME-Preserve or metabolic byproducts produced by other anaerobes in the community; however, the fact that we could recover the organism from fresh stool and from the T0 BIOME-Preserve tube (albeit at a much lower abundance than from stool) suggests that something native to BIOME-Preserve or to

community dynamics occurring over the span of days impacted this organism. It is possible that different media choice, different dilution factor(s), and/or different culture protocols may have successfully recovered this organism.

Recently, Martinez *et al.* [12] reported a sample collection and transport device (GutAlive) designed to maintain viability of extremely oxygen sensitive (EOS) bacteria common in human stool. They report higher bacterial diversity from GutAlive than a single “traditional” stool collection device (150ml polypropylene container), concluding that GutAlive is a better approach to conventional stool collection devices. Because Martinez *et al.* did not compare either stool collection device to the bacterial community of fresh stool (i.e., the gold standard), their protocol was unable to determine the extent to which GutAlive can preserve whole stool communities and there is no way to determine the true magnitude of loss of anaerobic species using GutAlive.

In contrast, we demonstrated that nearly 85% of species successfully cultured from fresh stool were still able to be cultured after 5 days in BIOME-Preserve, suggesting that not only does a collection device specifically tailored for anaerobes perform better than traditional stool collection devices as Martinez *et al.* showed, but also demonstrating that BIOME-Preserve specifically is an attractive option for collecting, storing, and recovering the majority of anaerobes that can also be cultured from human stool.

Unlike other similar studies, we did not protect the original fecal sample from oxygen between time of production until it arrived in the anaerobic chamber for homogenization, which may have some detrimental effects on extremely oxygen sensitive organisms. Notably, while the samples from donors 1 and 3 were transferred to the anaerobic chamber within 1 hour, the sample from donor 2 was not transferred to the anaerobic chamber until 4 hours after it was produced. Assessing extremely oxygen sensitive species (EOS) [28–30], we found that we successfully cultured several, including *Clostridium phoeensis* (which was actually recovered at a higher relative abundance from cultured BIOME-Preserve samples than from cultured stool) and *F. prausnitzii* [25, 31]. Other EOS were not successfully recovered, but as they were already low abundance in fresh stool (<0.05%) it is difficult to determine whether failure to culture is a result of oxygen damage or increased difficulty in capturing very low abundance organisms from a diverse microbial community. Our data suggest that our experimental procedures prior to anaerobic chamber work did not pose a significant detriment to our ability to capture anaerobic organisms generally; nevertheless, we recommend that for the best results, fecal material be transferred to BIOME-Preserve tubes as soon as possible after collection or otherwise protected from oxygen if immediate transfer to BIOME-Preserve isn't possible.

Conclusions

Altogether, our results show that BIOME-Preserve is an effective tool for collecting and preserving human stool samples for culture recovery of anaerobic organisms in the laboratory. The ability of BIOME-Preserve to culture over 85% of bacteria also cultured from fresh stool after five days at room temperature demonstrates the utility of this project for any microbiome study unable to immediately freeze or process samples, and will likely facilitate studies that until now have been infeasible.

Supporting information

S1 Fig. Photographs of representative plates showing culture density on A) plates used to count CFUs (low density) and B) high-density growth plates.
(TIF)

S2 Fig. Photographs of representative plates from a pilot study showing a roughly 2-fold reduction in CFU in BIOME-Preserve compared to fresh stool. A) 10^{-8} dilution of fresh stool, B), BIOME-Preserve, 10^{-6} dilution from fresh stool. This data informed the design of the current study, in we which inoculated the 10^{-3} dilution from BIOME-Preserve samples onto solid media plates but the 10^{-5} dilution from fresh stool.

(TIF)

S3 Fig. Stacked bar charts illustrate the relative abundances of bacterial phyla detected via BoosterShot whole-genome shotgun metagenomics sequencing in stool samples from all donors.

(TIF)

S1 Table. Sequence metrics for BoosterShot sequencing. Metrics include the number of reads per sample, the number of reads that mapped to a bacterial genome, and number of reads mapping to genus, species, and strain level.

(XLSX)

S2 Table. Sequence metrics for the six samples that underwent DeepSeq sequencing. Metrics include the number of reads per sample, the number of reads that mapped to a bacterial genome, and number of reads mapping to genus, species, and strain level.

(XLSX)

S3 Table. Rarefied (10,000 reads/sample) OTU table used in all downstream analyses.

Please note that this table contains relative abundances, not absolute counts. This formatting is automatically done during the data analysis process performed at CoreBiome and is delivered to the customer in this format.

(XLSX)

S4 Table. List of all species recovered, from high and low density media plates of fresh stool, at 0.01% relative abundance according to BoosterShot whole-genome shotgun metagenomics sequencing data. The unique region coverage and whole genome coverage (percentages) according to DeepSeq whole-genome shotgun metagenomics sequencing data are also listed.

(XLSX)

S5 Table. List of all species recovered, from high and low density media plates of stool held in BIOME-Preserve for 72 hours, at 0.01% relative abundance according to BoosterShot whole-genome shotgun metagenomics sequencing data. The unique region coverage and whole genome coverage (percentages) according to DeepSeq whole-genome shotgun metagenomics sequencing data are also listed.

(XLSX)

S6 Table. List of all species recovered, from high and low density media plates of stool held in BIOME-Preserve for 120 hours, at 0.01% relative abundance according to BoosterShot whole-genome shotgun metagenomics sequencing data. The unique region coverage and whole genome coverage (percentages) according to DeepSeq whole-genome shotgun metagenomics sequencing data are also listed.

(XLSX)

S7 Table. Species, and their relative abundances, detected in fresh stool from donor 1.

(XLSX)

S8 Table. Species, and their relative abundances, detected in fresh stool from donor 2.
(XLSX)

S9 Table. Species, and their relative abundances, detected in fresh stool from donor 3.
(XLSX)

S10 Table. Successfully cultured species are stratified by media type. Both the maximum detected relative abundance of a species on each media type as well as the total number of replicate plates of a single media type on which growth of a species was detected are listed.
(XLSX)

S11 Table. All successfully cultured species are listed, along with the number of replicate plates on which growth was detected. High and low density plates, stratified by media type, represent all time points. Additional columns break down numbers per time point per media type.
(XLSX)

S12 Table. The maximum relative abundance of each species cultured from fresh stool, BIOME-Preserve held at RT for 72 hours, and BIOME-Preserve held at RT for 72 hours and then frozen at -80°C for one week prior to culture recovery. Three replicates were plated for each sample, but only the maximum relative abundance as determined via WGS sequencing is listed and therefore only one replicate is represented in this table.
(XLSX)

S13 Table. The maximum relative abundance of each species cultured from fresh stool, BIOME-Preserve held at RT for 96 hours, and BIOME-Preserve held at RT for 96 hours and then frozen at -80°C for one week prior to culture recovery. Three replicates were plated for each sample, but only the maximum relative abundance as determined via WGS sequencing is listed and therefore only one replicate is represented in this table.
(XLSX)

S14 Table. The maximum relative abundance of each species cultured from fresh stool, BIOME-Preserve held at RT for 120 hours, and BIOME-Preserve held at RT for 120 hours and then frozen at -80°C for one week prior to culture recovery. Three replicates were plated for each sample, but only the maximum relative abundance as determined via WGS sequencing is listed and therefore only one replicate is represented in this table.
(XLSX)

S15 Table. Rarefied (10,000 reads/sample) OTU table obtained for the preliminary experiment used to assess the performance of BIOME-Preserve after being stored at -80°C. Please note that this table contains relative abundances, not absolute counts. This formatting is automatically done during the data analysis process performed at CoreBiome and is delivered to the customer in this format.
(XLSX)

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