GutAlive® provides easy self-collection and stabilization of the microbiome without disrupting the original composition and diversity

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Introduction

The microbiome is being extensively characterized, based on sequencing technologies coupled with bioinformatic pipelines, to decode the correlation between specific bacterial taxa and health status. A comprehensive knowledge of the taxa, relative abundance, metabolic pathways, and genetic features are key items to understand the microbial dynamics and crosstalk interactions with the host.

Oral, skin, or even vaginal microbiomes can be easily sampled whereas the gut microbiome represent a challenge with faecal samples being used as the gold standard that can represent intestinal microbiota to avoid invasive procedures[1].

Accurate and precise faecal microbiome analysis requires reliable and normalized methods of microbiome sampling, transport, and manipulation, to obtain a representative snapshot of the microbial community contained in each sample at the time of collection. Up to date, several kits are commercially available from various vendors, mainly focused on nucleic acid stabilization using specific buffers. However, these kits have limited volume size introducing bias over the self-collection sampling process of the donor and limitations on the amount of sample collected. Moreover, these kits do not consider microbial viability for downstream isolation of bacteria of interest, nor reliable sample conservation for faecal material transplant procedures.

GutAlive® is the unique microbiome collection kit that generates an anaerobic atmosphere enabling oxygen sensitive bacteria to survive, maintaining the original composition and diversity of the microbiota[2] making it suitable for various downstream applications. It enables the

standardization of sample collection and transport, without refrigeration, minimizing variations among procedures and maintaining the original composition of the microbiota, key items for diverse applications like metagenomics analyses, diagnostics, anaerobic bacterial isolation, microbiota-based therapeutics, and faecal microbiome transplant[2].

In this study we demonstrate that GutAlive® is a suitable device to stabilize, at room temperature, the faecal microbiome community over time, long enough for sample collection and delivery to perform DNA extraction and metagenomics analyses, in addition to its already demonstrated capability of maintaining microbial viability.

Material and Methods

Sample Collection and DNA extraction

Four healthy adult donors were asked to collet one faecal sample and divide it in four different samples for self-collection using GutAlive® kits, generating a total of 16 samples. All the samples were shipped to Microviable laboratory facilities at room temperature and received within 24 hours of collection. The samples were kept at ambient temperature (15ºC-25ºC) and processed at 24, 48, 72 and 120 hours from collection, as described below (Figure 1).

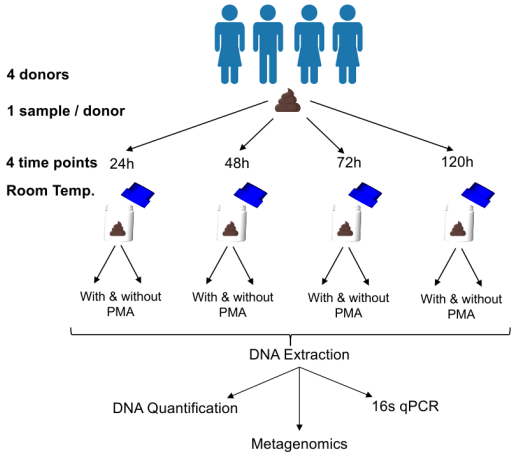

Figure 1. *Experimental design for faecal sample collection using GutAlive and different processing time points to elucidate bacterial viability, DNA stability and downstream applications like microbiome analyses.*

For each sample and time point, 600 mg of faeces were homogenized and divided in two aliquots of 300 mg for DNA extraction, with and without Propidium Monoazide (PMA) treatment. PMA allows differentiation between intracellular DNA (from viable bacteria) and extracellular free-DNA (dead bacterial cells) in the subsequent PCR amplifications.

Briefly, for the PMA-treated aliquot, the 300 mg of faeces were resuspended in 800 µl of PBS and 2 µl of PMA were added, followed up by 10' incubation in darkness, 30' incubation under blue light and 5' centrifugation at 15,000 rpm. Then, DNA extraction was performed using QIAamp® PowerFecal® Pro DNA Kit (Qiagen).

The extracted DNA was quantified with Qubit Fluorometric dsDNA quantification

kit and with Nanodrop (260 nm, 260/280 nm ratio).

Real-time qPCR

The extracted DNA was amplified using 16s rRNA real-time PCR (qPCR) in order to validate PCR efficiency, detect potential carry over inhibitors and to compare bacterial viability. The primers used were qPCR1369-F (5'CGGTGAATACGTTCCCGG3') and qPCR1492-R (5'TACGGCTACCTTGTTACGACTT3')[3]. The correlation between DNA quantity and total or viable cells (cfu/gr.) was made using *Escherichia coli* DNA for the standard curve. Experiments were performed in triplicates, with 10-fold serial dilutions ranging from 10^3 cfu and 10^7 cfu of *E. coli* DNA were carried out to generate the standard curves. All real-time PCR runs were performed on HT7900 Real Time PCR equipment (Applied Biosystems) with a default protocol in a Standard mode. The amplification reaction was made in a final volume of 20µl containing 1X of Power Syber® Green PCR Master Mix (Thermo Scientific), 300nM of each oligonucleotide and 0.5 ng of total DNA, PMA treated or not, from each sample and collection time point, in triplicate. Equal amounts of DNA of each sample and time point were used and correlation with ng of DNA and grams (gr.) of faecal samples were calculated.

The Ct values were obtained using software Sequence Detection System (SDS) v2.3 (Applied Biosystems), and the CFU were calculated by y-intercept and slope from the standard curve, as described: Cell number_sample = (Ct - y-intercept) / slope).

Shotgun Metagenomics

To evaluate the microbiome profiling, DNA samples were sequenced using shotgun metagenomics on an Illumina HiSeq 2500 with paired-end 150 bp reads, at Eurofins Scientific (Ebersberg, Germany).

The distribution of sequenced reads per sample varied between 19,436,024 and 33,248,090. After quality filtering with KneadData, sequencing reads were between 18,191,103 and 31,163,678. The number of sequences to be used was rarefied adjusted to the minimum number of sequences to avoid biases due to the different quantity of information (number of sequences) per sample. The quality-filtered sequencing reads were used for the identification and taxonomic assignment of the bacterial groups present in the samples, using MetaPhlAn 3.0 and the CHOCOPhlan v201901 database. After that, relative abundances of each taxon with respect to the total were calculated.

Statistical analysis (alpha- and beta-diversity, and Analysis of Composition of

Microbiomes) were performed in R-Studio 2022.02.1+461 for Ubuntu 22.04.3 LTS environment.

Results

Convenient device

The self-collection kit for microbiome sample and transport in anaerobic conditions, GutAlive®, provides a user-friendly option for microbiome analysis, metagenomics, diagnostics, and other downstream applications like FMT and bacterial isolation including strict anaerobic bacteria[3].

GutAlive® enables the standardization of sample collection and transport, with no limitations regarding to sample size, as this unique device can fit up to 120 grams of stool sample.

The high capacity of the device represents an advantage compared to other commercially available small collection tubes (2ml-5ml) only focused on nucleic acid stabilization. With this high-capacity container, there is no bias in sample collection, performed by the donor, and no limitation on the amount collected, representing an advantage in case several applications with the same sample are going to be performed.

DNA quantity and quality

GutAlive® enables microbiome sampling preserving bacterial viability together with DNA stability and integrity, which are guarantee.

High-yield DNA extractions were obtained for all the analysed samples, independently of each donor and sampling point. An average of 443 ± 208 ng/µl or 449±137 ng/µl was obtained (Qubit Fluorometric dsDNA quantification kit or Nanodrop, respectively), when 300 mg of faecal sample was used for DNA extraction and total DNA was eluted in 100 µl. No significant differences between samples, treatments or time points were observed (Figure 2).

The quality (260/280 nm) of the extracted-DNA was 1.80 ± 0.02 with no significant differences between samples or time points.

GutAlive® enables DNA stability and integrity, representing a suitable device for microbiome sampling with no limitation on DNA quality and quantity for next generation sequencing technologies and microbiome analyses through 16s rRNA sequencing or metagenomics.

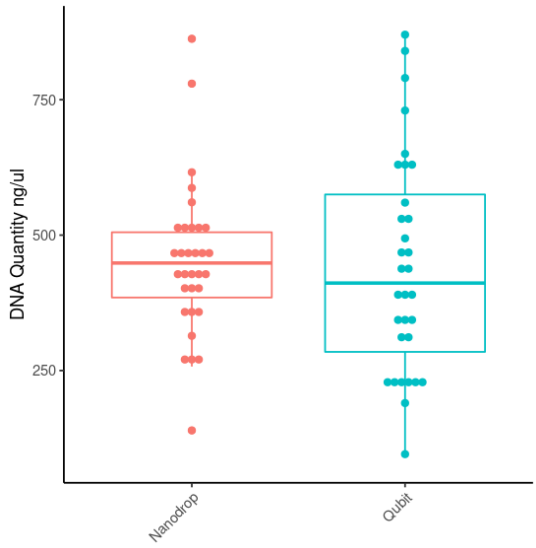

Figure 2. *Quantification of total DNA extractions from stool sample collected with GutAlive®*

Real time qPCR analyses

The use of GutAlive® for microbiome sampling coupled with the QIAamp® PowerFecal® Pro DNA Kit and quantitative PCR (qPCR) enables precise bacterial quantification.

Bacterial DNA quantity, integrity and quality has shown no limitation for qPCR analyses for microbial population quantification (16S rRNA primers) when stool samples are collected using GutAlive®. No DNA degradation nor inhibitors that impede qPCR amplification were detected, with GutAlive-sampling strategy displaying the same DNA quality and applicability than other commercially available DNA preservation collection devices.

The average of total bacteria detected (DNA without PMA) was 11,40±0,10 CFU/gr (Log10, Mean±SD), and viable bacteria was 11,30±0,17 CFU/gr, displaying no significant differences (Figure 3A). The absence of differences showcases that GutAlive® preserves bacterial viability and therefore, bacterial DNA is in good condition for qPCR microbial quantification and other DNA-based approaches.

Moreover, no significant differences were detected across the five days of the study (four time points of sample processing), validating bacterial viability, DNA-preservation and stability (Figure 3B). A minor reduction of 0.4 log units on bacterial viability was detected after 5 days of storing the sample in GutAlive® at room temperature.

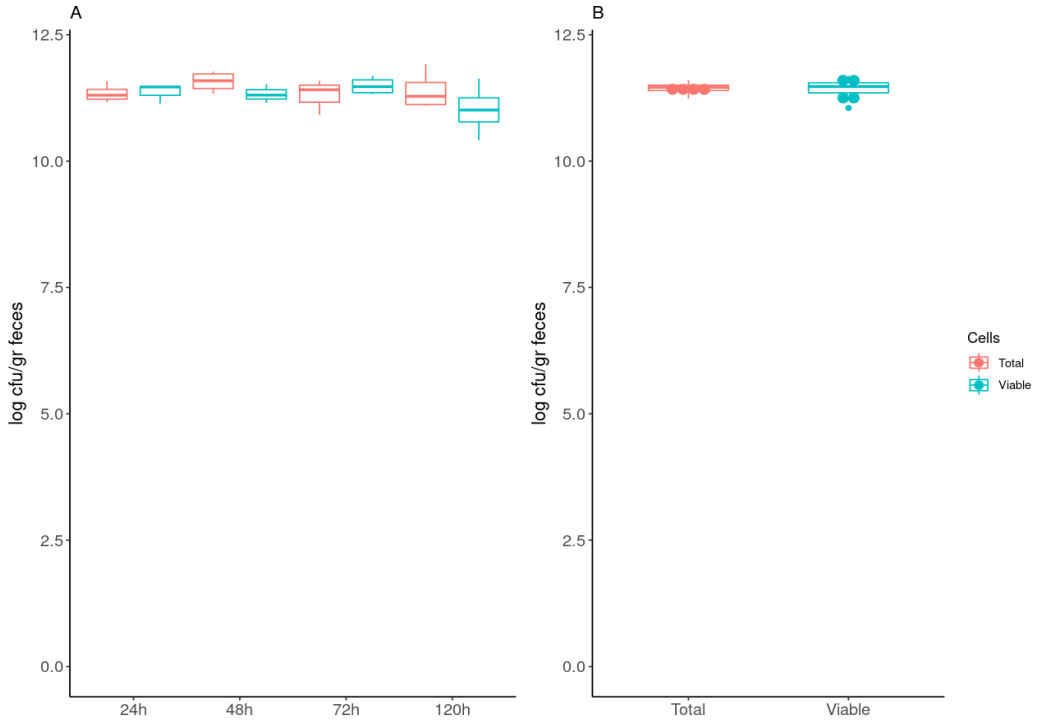

Figure 3. *Bacterial quantification over the five days of the study, using 16s rRNA qPCR. Total bacteria and viable bacteria were distinguished by the addition of PMA at the time of DNA extraction*.

Microbiome analysis

The use of GutAlive® for microbiome sampling enables shotgun metagenomics analyses without disrupting the microbial population over time.

The alpha diversity, quantified with Shannon index, did not show significant differences over time (p-value>0.05), displaying that the identified bacterial diversity was stable over the five days of study (Figure 4A).

The beta diversity analyses with Bray-Curtis Dissimilarity index, taking in account both the presence-absence of bacterial taxonomic units and their relative abundance, displayed a differential clustering (Adonis p-value <0.001) of the samples based on the donor microbiome profiling but no influence of the sampling point of analyses (Figure 4B). These results displayed that independently of the original microbiome composition of each donor, the microbial population remains stable overtime. Moreover, individuals can be distinguished based on their microbiome profiling at any given time point.

These results showcase that GutAlive® was able to maintain sample stability over the five days of the study, capturing a snapshot of the original microbiome composition and diversity that represents a fingerprint of each donor over time.

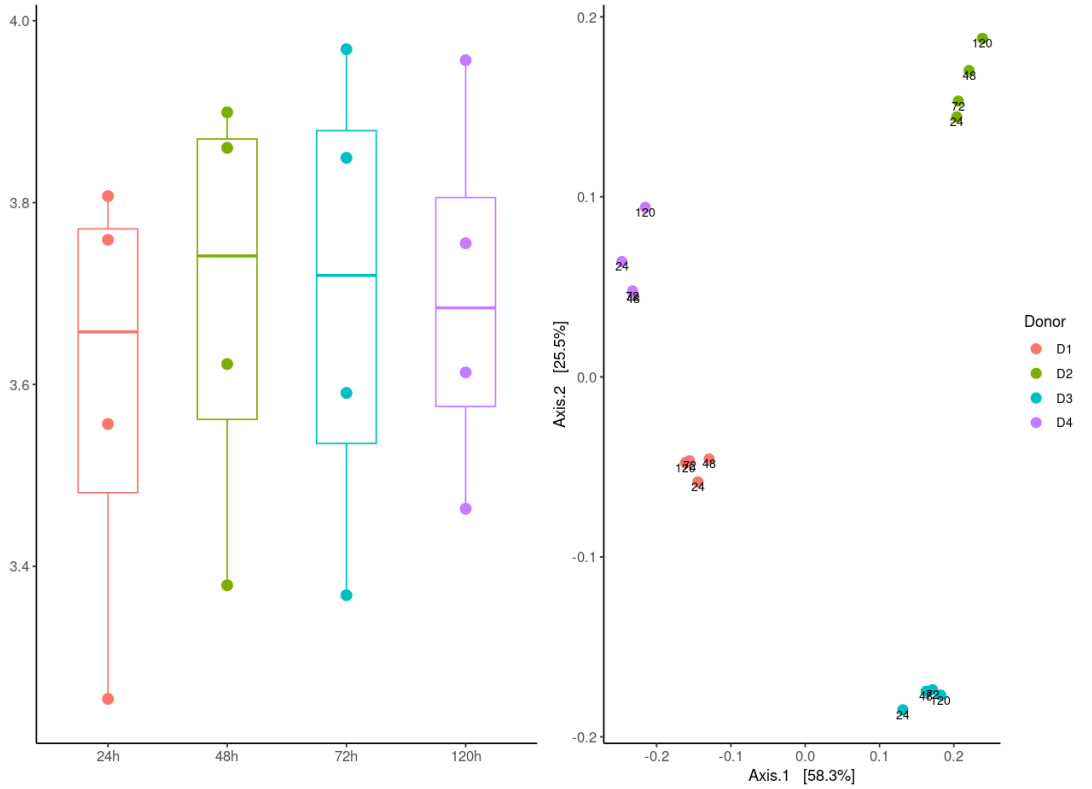

Figure 4. *Shannon Index boxplot (A) and PCoA representation of Bray Curtis distance (B).*

For a deeper visualization of potential microbiome fluctuations, ANCOM statistical analyses at family level taxonomic unit were performed for each individual donor between each time point. No significant differences in relative abundance were detected over the five days of the study, reflecting the stability of the sample for each individual donor.

These results displayed that GutAlive® enables sample stabilization with no significant alteration of the microbiome composition of everyone over time, regardless of the original microbiome profiling (Figure 5).

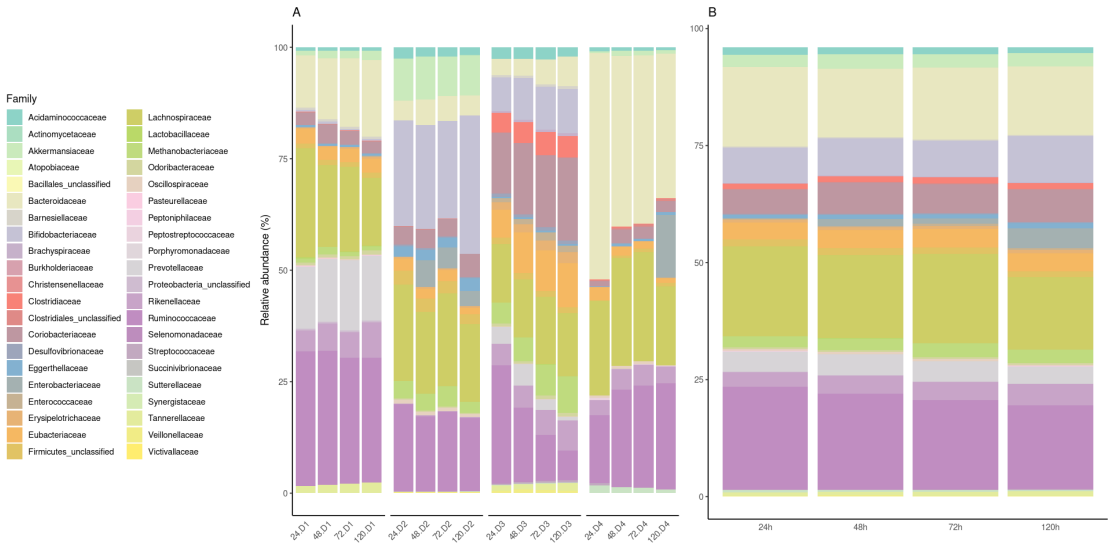

Figure 5. *Family-level bacterial relative abundance represented as stacked bar plot of four donors at different sample processing times (A) and average of the donors (B).*

Conclusions

GutAlive® is a unique on its kind device for **self-collection of stool samples in anaerobic conditions that enables microbiome analyses**, bacterial isolation and FMT applications thanks to its **capability of preserving bacterial viability**.

GutAlive® enables **high-yield, high-quality, high-purity** DNA extraction **for downstream applications including qPCR, NGS platforms and microbiome analyses**.

GutAlive® enables **microbiome samples stability over time** preserving the original composition and diversity of the faecal microbiome, with no alterations (alpha- and beta-diversity) of the microbial populations.

GutAlive® captures a **snapshot of each individual** microbiome as a fingerprint, preserving its composition over time, regardless of their original microbial populations.

References

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