Příprava vzorků – odběr vzorků, uchování vzorků a izolace

Petra Vídeňská, Ph.D.

Workflow

Design studie

↓

Odběr vzorků

↓

Izolace DNA

↓

Příprava knihovny ↗ Amplikony – 16S rRNA

Příprava knihovny ↘ Shot gun – celometagenomové sekvenování

↓

Sekvenace

↓

Analýza

Faktory ovlivňující výsledek sekvenace genu pro 16S rRNA

- Sběr vzorků
  - Čas vystavení vzduchu
  - Teplota
  - Postup
- Izolace DNA
  - Použitý kit a metoda disrupce bakteriálních buněk
  - Čas po který je vzorek rozmrazován před izolací
- Příprava knihovny
  - Výběr primerů
  - Polymeráza
  - Počet cyklů PCR (vznik chimér)
- Sekvenace
  - Výběr sekvenační platformy
  - Kvalita knihovny
- Analýza
  - Použitý quality trimming
  - Použité algoritmy pro výběr OTU
  - Algoritmus výběru reprezentativních sekvencí
  - Výběr databáze 16S rDNA

Nejčastější typy vzorků

- Voda
- Půda
- Biologické vzorky
  - Stolice
  - Sliny
  - Stěry

!Nejdůležitější je vždy zachovat stejný postup odběru!

Odběr vzorků - půda

- Vhodně zvolit lokalitu, možno i z několika hloubek a vícero opakování vzorkování
- Odběr do sterilní nádoby, sáčku
- Transport na ledu, následné zmražení na -20 °C až -80°C
- Na izolaci většinou postačí malé množství půdy, často se ale měří vícero hodnot a odebírá se tedy velké množství – půdu je před zpracováním nutno homogenizovat

Odběr vzorků - voda

- Možno odebírat z více hloubek ve zvolené lokalitě
- Odebírá se větší objem (dle očekáváné bakteriální kontaminace 100 – 1000 ml) a transportuje se na ledu do laboratoře
- Následně se voda filtruje přes bakteriologický filtr
- Filtr je vložen do vhodné tekutiny (voda, TE, PBS) a promyje se

Odběr vzorků – biologické vzorky

- složitější, např. u stolice velké množství anaerobů – fakultativně anaerobní bakterie rychle přerůstají
- Nutno okamžitě zamrazit
- Pokud není možno – komerční kity s pufry sloužící ke stabilizaci DNA a zamezení přerůstání bakterií po dobu XY dnů

Odběr vzorků stolice - problémy

- Nestandardizované odběry vzorků – různá délka vystavení vzorku kyslíku a teplotám před zamrazením
- Různé formy stolice (průjem, pevná stolice, dětská stolice, mekonium) – různé typy odběrových nádob
- Problém homogenizace – odběr jen z jednoho místa stolice
- Přítomnost inhibitorů PCR

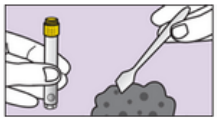
Komerční souprava na odběr vzorků stolice

OMNIgene•GUT (OMR-200)

1

Unscrew the purple cap from the collection device and set it aside for later use.
IMPORTANT:
Do NOT remove the yellow tube top.
Do NOT spill the stabilizing liquid in the tube.

2



Use the stick to collect a small amount of fecal sample.

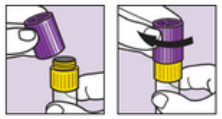
3

Transfer the fecal sample into the yellow tube top
Repeat until the sample reaches the top and fills it completely.
IMPORTANT: Do NOT push sample into the tube.

4

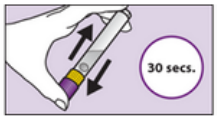
Scrape horizontally across the tube top to level the sample and remove any excess. Discard the stick. Wipe exterior of tube and top with toilet paper or tissue as needed.

5



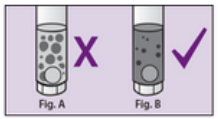
Screw the purple cap back onto the yellow tubetop until tightly closed.

6



Shake the sealed tube as hard and fast as possible in a back and forth motion for a minimum of 30 seconds.

7



The fecal sample will be mixed with the stabilizing liquid in the tube; not all particles will dissolve.
IMPORTANT: Continue shaking if large particles remain as shown in Figure A.
Send the sample for processing following the delivery instructions supplied by the kit provider.

Komerční souprava na odběr vzorků stolice

- ✔ Nucleic Acid Preservation (at ambient temperature; cold-free)
- ✔ Pathogen Inactivation (bacteria, fungus, parasites & viruses)
- ✔ Streamlined Purification (no reagent removal, universally compatible, automatable)

DNA/RNA Shield - Fecal Collection Tube

DNA/RNA transport medium for stool samples

Size 10 Pack

10 Pack

SKU IN STOCK

R1101

1

For pricing, please contact your distributor

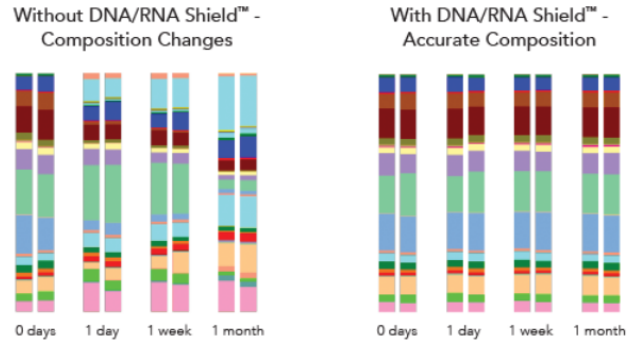


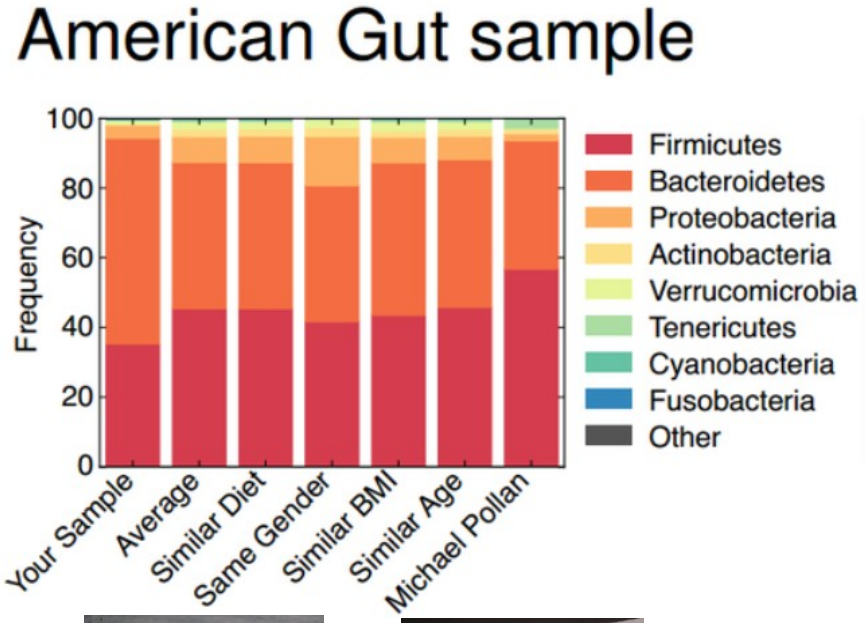
Figure 4: Microbial composition of stool samples preserved by DNA/RNA Shield ™ versus samples left unprotected. Microbial composition (indicated by differe stool samples were stored over time at ambient temperature with and without by DNA/RNA Shield ™. DNA was extracted with ZymoBIOMICS® DNA Miniprep subjected to 16S rRNA targeted sequencing. Want to try it out for yourself? Cli request a sample of DNA/RNA Shield.™

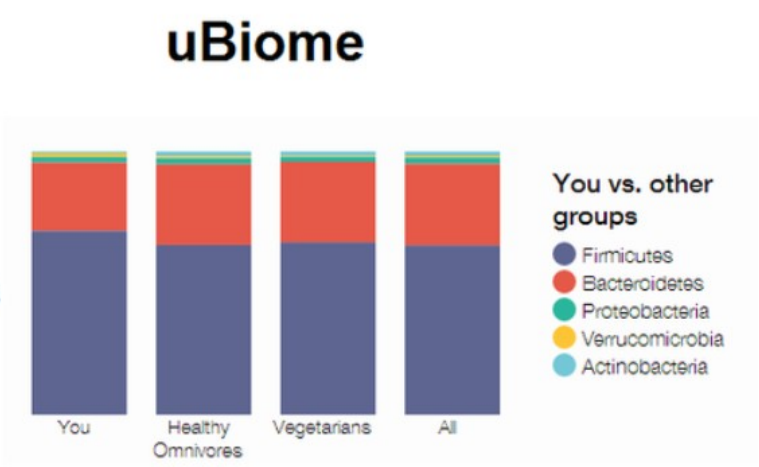
Komerční kity

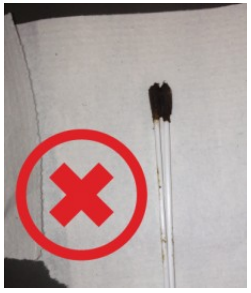
- Jednoduché workflow, ale málokdy se hodí na všechny typy stolice
- Pozor na bezpečnost – obsahují chemikálie, vždy v návodu uvádějte i informace o bezpečnosti (co dělat když se člověk potřísní nebo pokud semu dostane pufr do oka apod.)

Ukázka různých přístupů – American Gut Project x uBiome

- https://mrheisenbug.wordpress.com/2014/04/24/dear-american-gut-ubiome-you-have-some-explaining-to-do/







American Gut Project – podmínky skladování

- http://www.ncbi.nlm.nih.gov/pubmed/20412303

FEMS Microbiol Lett. Author manuscript; available in PMC 2011 August 1.

Published in final edited form as:
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Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples

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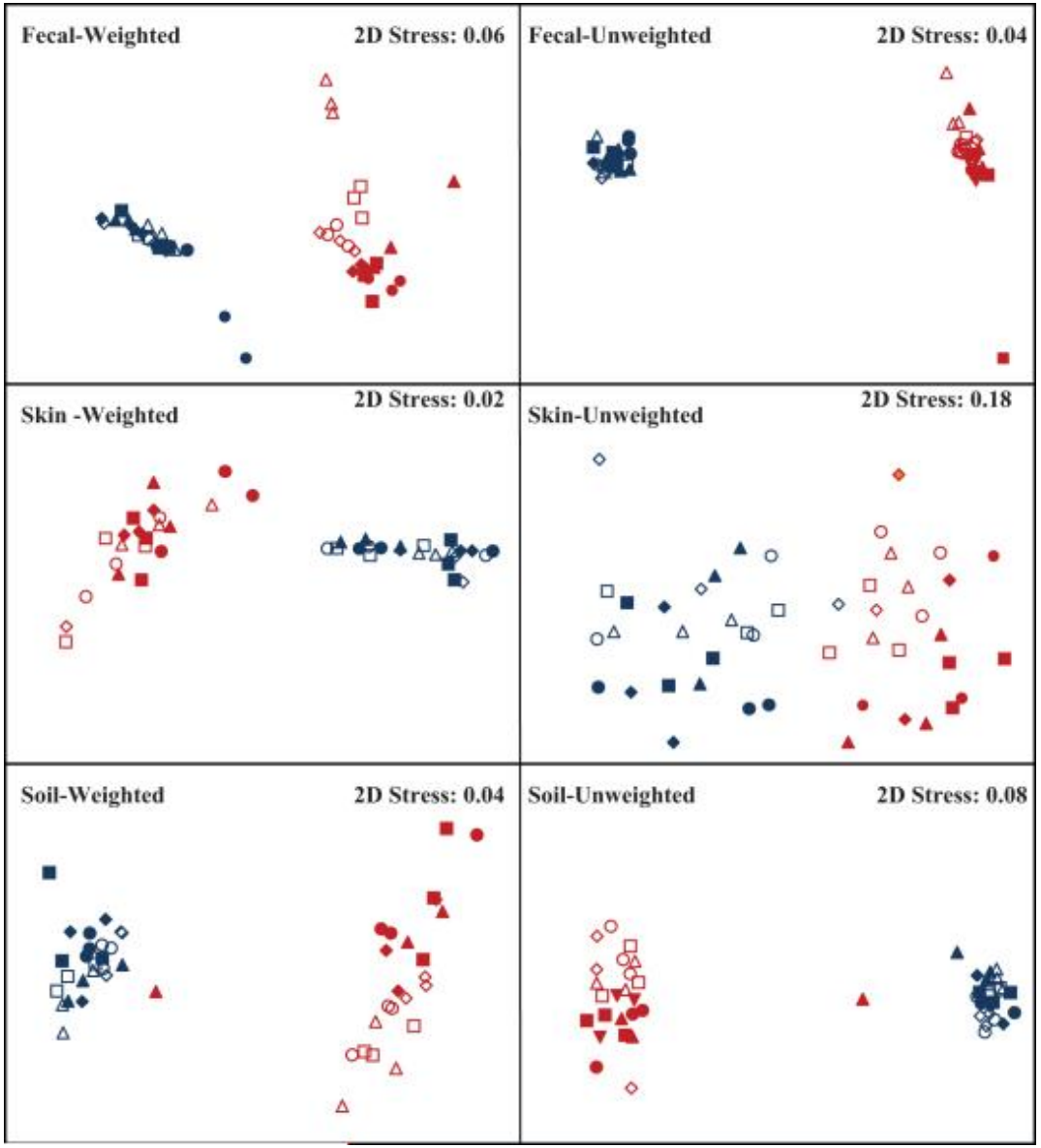
Abstract

Storage conditions are considered to be a critical component of DNA-based microbial community analysis methods. However, whether differences in short-term sample storage conditions impact the assessment of bacterial community composition and diversity demands systematic and quantitative assessment. Therefore, we used barcoded pyrosequencing of bacterial 16S rRNA genes to survey communities, harvested from a variety of habitats (soil, human gut (feces) and human skin) and subsequently stored at 20°, 4°, −20°, and −80°C for 3 and 14 days. Our results indicate that the phylogenetic structure and diversity of communities in individual samples was not significantly influenced by storage temperature or duration of storage. Likewise, the relative abundances of most taxa were largely unaffected by temperature even after 14 days of storage. Our results indicate that environmental factors and biases in molecular techniques likely impart greater amounts of variation to microbial communities than do differences in short-term storage conditions, including storage for up to two weeks at room temperature. These results suggest that many samples collected and stored under field conditions without refrigeration may be useful for microbial community analyses.

American Gut Project – podmínky skladování



Non-metric Multidimensional Scaling (NMDS) plots of UniFrac weighted and unweighted pairwise distances. Overall community composition was not affected by temperature or duration of storage for weighted UniFrac distances ($P > 0.1$ in all cases). Length of storage significantly affected the skin communities for the unweighted UniFrac metric ($P = 0.02$). The remaining unweighted UniFrac distances were not significantly different by day or temperature. Blue=sample 1, red = sample 2. Open symbols = Day 3, closed symbols = Day 14. ▲ = 20°C, 4°C, ● = −20°C, ◆ = −80°C.

Vliv laboratorní teploty na degradaci DNA

Cardona *et al. BMC Microbiology* 2012, **12**:158
http://www.biomedcentral.com/1471-2180/12/158

BMC Microbiology

RESEARCH ARTICLE Open Access

Storage conditions of intestinal microbiota matter in metagenomic analysis

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Abstract

Background: The structure and function of human gut microbiota is currently inferred from metagenomic and metatranscriptomic analyses. Recovery of intact DNA and RNA is therefore a critical step in these studies. Here, we evaluated how different storage conditions of fecal samples affect the quality of extracted nucleic acids and the stability of their microbial communities.

Results: We assessed the quality of genomic DNA and total RNA by microcapillary electrophoresis and analyzed the bacterial community structure by pyrosequencing the 16S rRNA gene. DNA and RNA started to fragment when samples were kept at room temperature for more than 24 h. The use of RNAse inhibitors diminished RNA degradation but this protection was not consistent among individuals. DNA and RNA degradation also occurred when frozen samples were defrosted for a short period (1 h) before nucleic acid extraction. The same conditions that affected DNA and RNA integrity also altered the relative abundance of most taxa in the bacterial community analysis. In this case, intra-individual variability of microbial diversity was larger than inter-individual one.

Conclusions: Though this preliminary work explored a very limited number of parameters, the results suggest that storage conditions of fecal samples affect the integrity of DNA and RNA and the composition of their microbial community. For optimal preservation, stool samples should be kept at room temperature and brought at the laboratory within 24 h after collection or be stored immediately at −20°C in a home freezer and transported afterwards in a freezer pack to ensure that they do not defrost at any time. Mixing the samples with RNAse inhibitors outside the laboratory is not recommended since proper homogenization of the stool is difficult to monitor.

Keywords: Needs for standardization/RNA and DNA degradation/Metagenomics/16S ribosomal RNA

Vliv laboratorní teploty na degradaci DNA

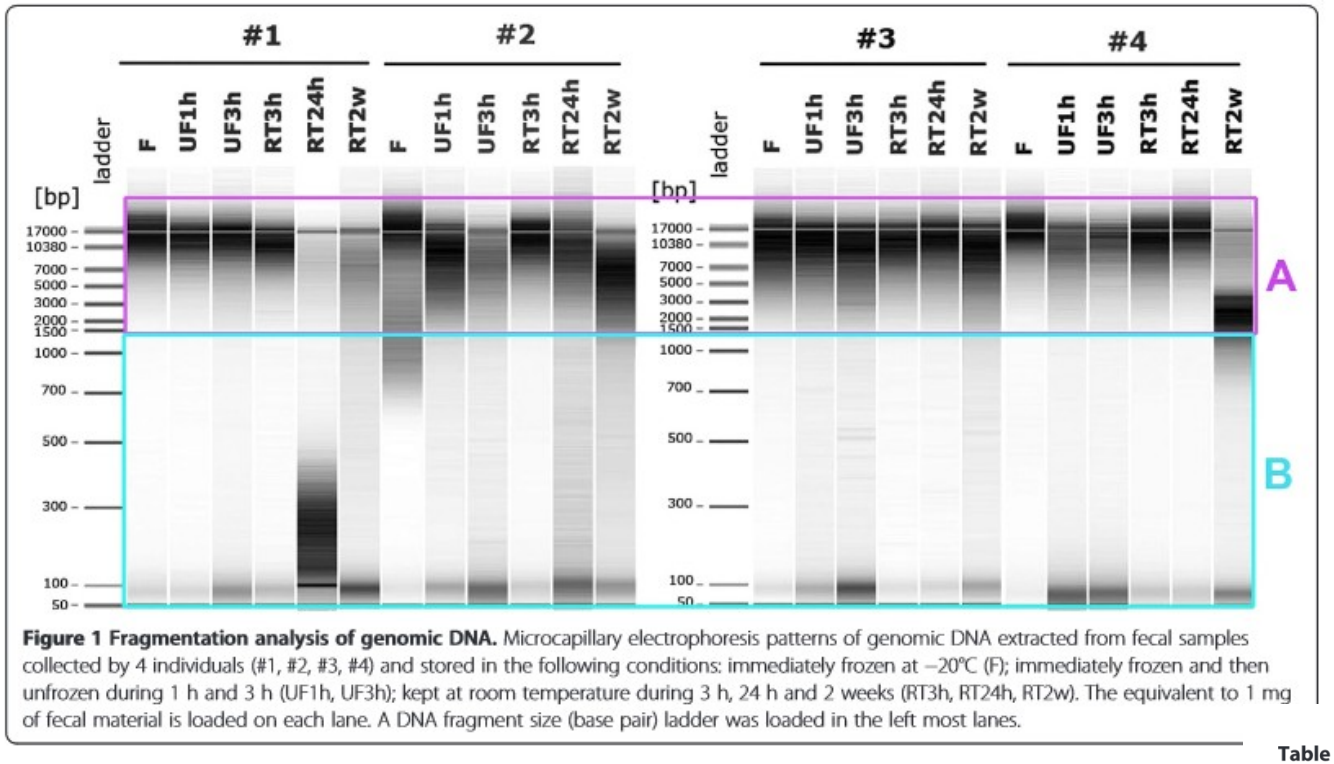

Figure 1 Fragmentation analysis of genomic DNA. Microcapillary electrophoresis patterns of genomic DNA extracted from fecal samples collected by 4 individuals (#1, #2, #3, #4) and stored in the following conditions: immediately frozen at −20°C (F); immediately frozen and then unfrozen during 1 h and 3 h (UF1h, UF3h); kept at room temperature during 3 h, 24 h and 2 weeks (RT3h, RT24h, RT2w). The equivalent to 1 mg of fecal material is loaded on each lane. A DNA fragment size (base pair) ladder was loaded in the left most lanes.

Table 1 Percentage of DNA compared to the frozen samples

	% degraded DNA				n = 4
	#1	#2	#3	#4	*p* value when compared to frozen samples
F	12	28	10	9	
UF1h	12	24	23	34	< 0.01
UF3h	25	39	31	34	< 0.001
RT3h	17	16	12	15	0.9270
RT24h	84	44	13	15	< 0.001
RT2w	48	38	26	40	< 0.001

Statistical analysis was performed using Poisson regression mo
value < 0.05 is considered significant; #1, #2, #3, #4 correspond
3, 4; F = frozen; UF1h = unfrozen during 1 h; UF3h = unfrozen during 3 h;
RT = room temperature; 2w = 2 weeks.

Vliv laboratorní teploty na degradaci DNA

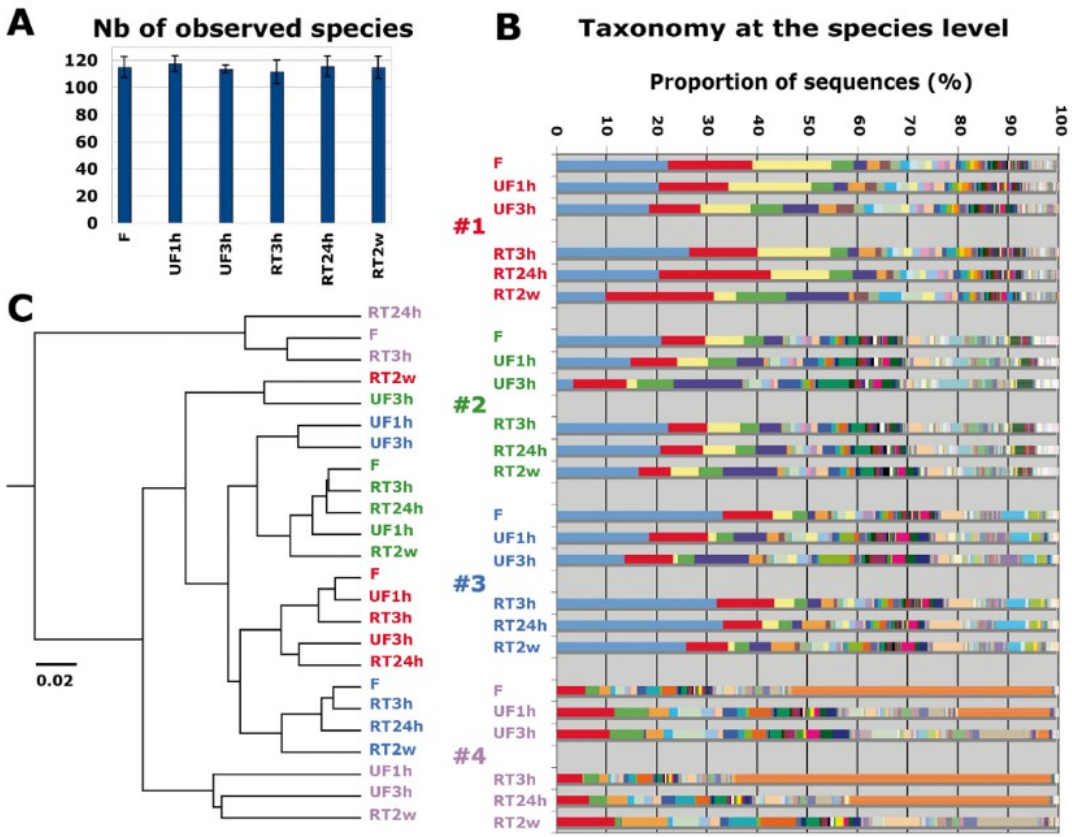

Figure 2 Bacterial community analysis based on 16S rRNA gene survey. A) Alpha-diversity analysis of number of species observed in 6 storage conditions: Immediately frozen (F); unfrozen 1 h and 3 h (UF1h, UF3h); room temperature 3 h, 24 h, and 2 weeks (RT3h, RT24h, RT2w). The plot averages the number of species from the samples provided by 4 individuals in each condition. **B**) Taxonomy analysis at the species level of the 24 samples based on alignment performed using PyNast against Silva 108 release database and OTUs assignment using blast and the Silva 108 release taxa mapping file. Individual #1 (red), #2 (blue), #3 (green), #4 (purple). A more detailed taxonomy assignment is provided in the additional data (See Additional file 3: Table S1). **C**) UPGMA clustering of the 24 samples based on weighted UniFrac method. Samples from the 4 individuals are colored as in B. The scale bar represents 2% sequence divergence.

Vliv laboratorní teploty na degradaci DNA

Table 2 Taxonomic comparison for 3 main bacterial taxa between frozen and unfrozen samples

Taxon	F*	UF1h*	UF3h*	*p* value F vs UF1h	*p* value F vs UF3h
Bacteroides;uncultured bacterium	19	13	9	0.044	9.68e-05
Prevotellaceae;uncultured;human gut metagenome	7	6	3	0.6804	0.0222
Bifidobacterium;uncultured bacterium	2	4	8	0.2257	0.0007

Statistical analysis was performed using Poisson regression model; p value < 0.05 is considered significant; n = 4 subjects; * Values are mean proportion of sequences (%).
F = frozen; UF1h = unfrozen during 1 h; UF3h = unfrozen during 3 h; Taxonomy is indicated at the genus level and if not possible at the family level.

Table 3 Taxonomic comparison for 3 main bacterial taxa between frozen and RT samples

Taxon	F*	RT3h*	RT24h*	RT2w*	*p* value F vs RT3h	*p* value F vs RT24h	*p* value F vs RT2w
Bacteroides;uncultured bacterium	19	20	19	13	0.749	0.749	0.0349
Prevotellaceae;uncultured;human gut metagenome	7	6	5	3	0.6804	0.3189	0.0140
Bifidobacterium;uncultured bacterium	2	2	3	7	1	0.3964	0.0030

Statistical analysis was performed using Poisson regression model. * Values are mean proportion of sequences (%). p-value < 0.05 is considered significant; n = 4 subjects; F = frozen; UF1h = unfrozen during 1 h; UF3h = unfrozen during 3 h; RT = room temperature; 2w = 2 weeks; Taxonomy is indicated at the genus level and if not possible at the family level.

Vliv skladovacích podmínek na bakteriální složení vzorku

SCIENTIFIC REPORTS

OPEN

Sample storage conditions significantly influence faecal microbiome profiles

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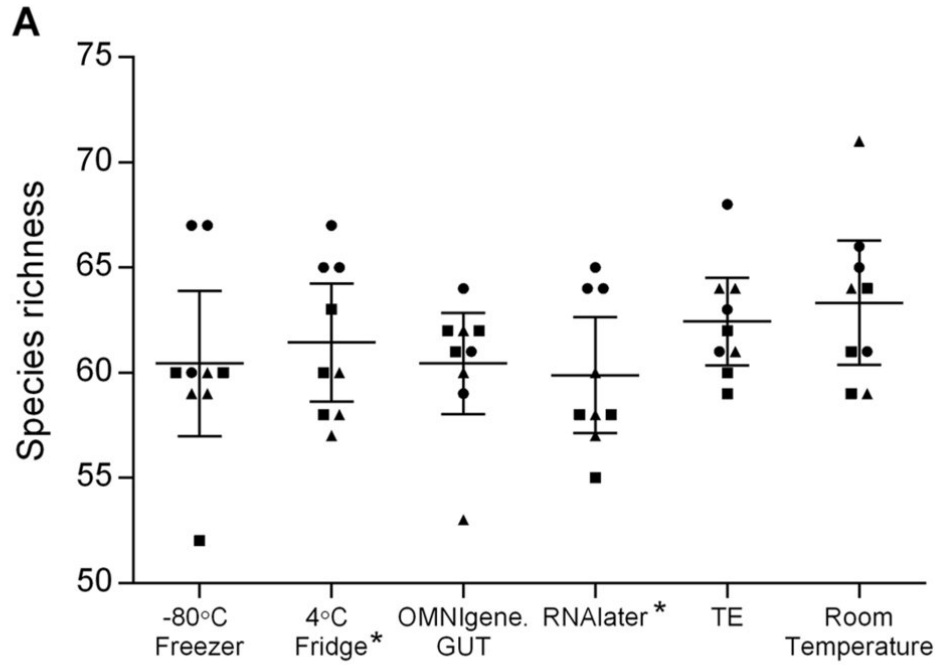
Jocelyn M Choo[1,*], Lex EX Leong[1,*] & Geraint B Rogers[1,2]

Sequencing-based studies of the human faecal microbiota are increasingly common. Appropriate storage of sample material is essential to avoid the introduction of post-collection bias in microbial community composition. Rapid freezing to −80 °C is commonly considered to be best-practice. However, this is not feasible in many studies, particularly those involving sample collection in participants' homes. We determined the extent to which a range of stabilisation and storage strategies maintained the composition of faecal microbial community structure relative to freezing to −80 °C. Refrigeration at 4 °C, storage at ambient temperature, and the use of several common preservative buffers (RNAlater, OMNIgene.GUT, Tris-EDTA) were assessed relative to freezing. Following 72 hours of storage, faecal microbial composition was assessed by 16 S rRNA amplicon sequencing. Refrigeration was associated with no significant alteration in faecal microbiota diversity or composition. However, samples stored using other conditions showed substantial divergence compared to −80 °C control samples. Aside from refrigeration, the use of OMNIgene.GUT resulted in the least alteration, while the greatest change was seen in samples stored in Tris-EDTA buffer. The commercially available OMNIgene.GUT kit may provide an important alternative where refrigeration and cold chain transportation is not available.

Vliv skladovacích podmínek na bakteriální složení vzorku

Figure 1: Species diversity following incubation under six different storage conditions

From: Sample storage conditions significantly influence faecal microbiome profiles

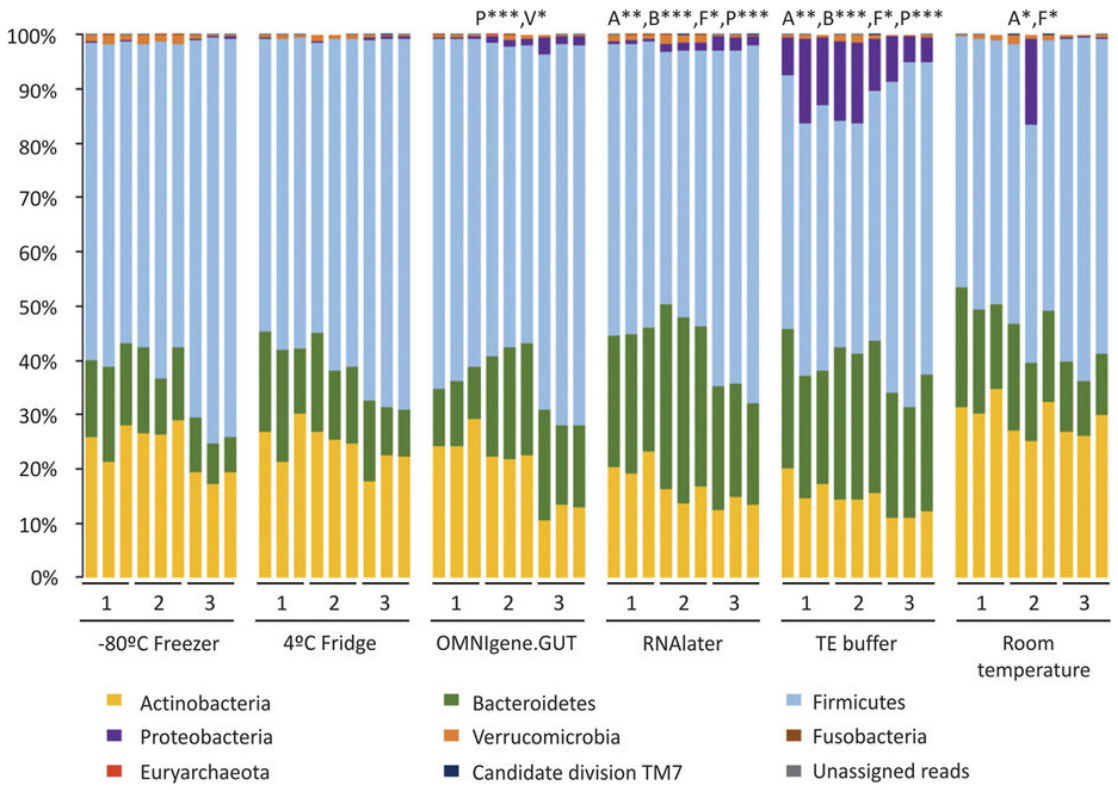


The extent of microbiota structural and composition diversities were measured using (**A**) Taxa S (species richness), (**B**) Shannon-Weiner diversity index, (**C**) Simpson's evenness index. Each point represents the diversity score for a replicate from collection 1 (●), collection 2 (▲) or collection 3 (■). Error bars represent SEM. Within-group and between-group variations were measured using Kruskal-Wallis one-way ANOVA and Mann-Whitney *U*-test, respectively. Significant variance is indicated by asterisks; single asterisk (*) indicates $p \leq 0.05$ double asterisk (**) represe $p \leq 0.01$.

Vliv skladovacích podmínek na bakteriální složení vzorku

Figure 2: Relative abundance at phylum level for each sample incubated under six different storage conditions.

From: Sample storage conditions significantly influence faecal microbiome profiles



Storage conditions that differed significantly from the control (−80 °C) are indicated with respective phylum abbreviation and asterisks. The respective phyla were abbreviated as follow: Actinobacteria (**A**), Bacteroidetes (**B**), Firmicutes (**F**), Proteobacteria (**P**) and Verrucomicrobia (**V**). Statistical significance was assessed by Mann-Whitney U-test and indicated by asterisks; single asterisk (*) represents $p \leq 0.05$, double asterisk (**) represents $p \leq 0.01$, and triple asterisk (***) represents $p \leq 0.001$.

Vliv skladovacích podmínek na bakteriální složení vzorku

Table 1: Mean difference in the relative abundance of the phyla Firmicutes Bacteroidetes, Actinobacteria and Proteobacteria in different storage conditions compared to −80°C.

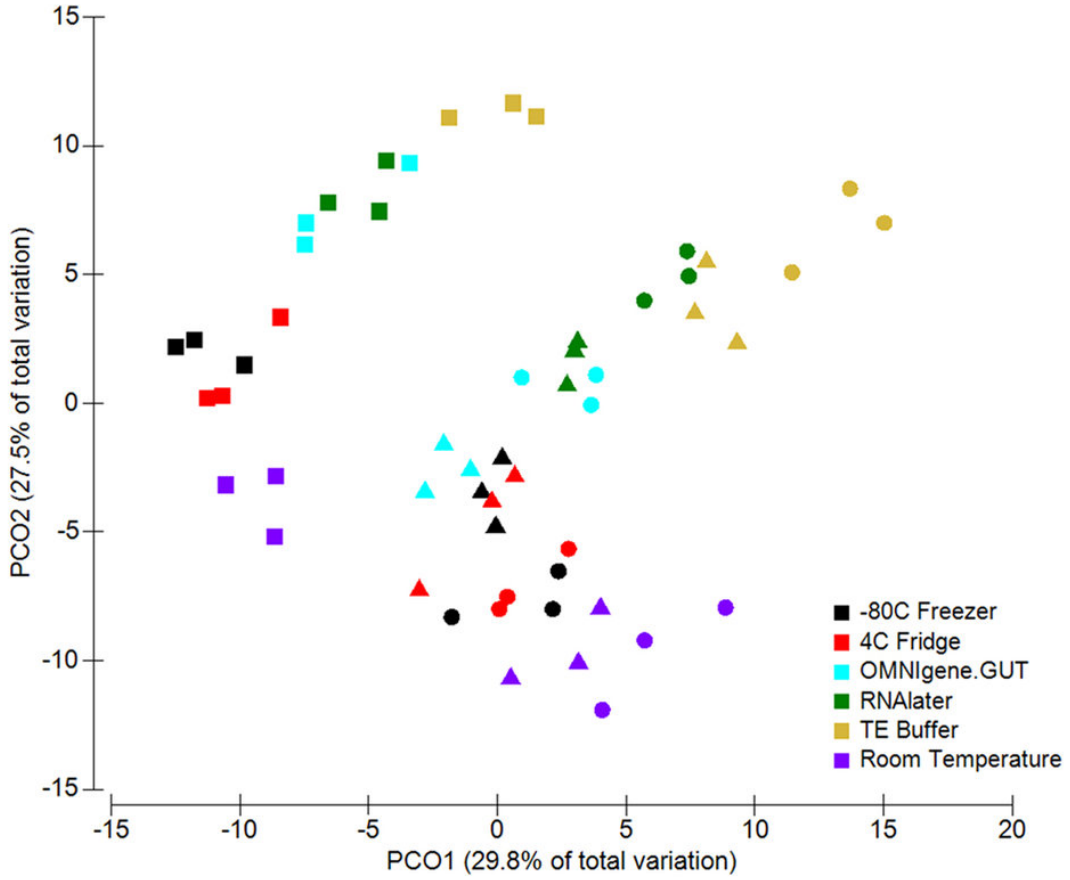
From: Sample storage conditions significantly influence faecal microbiome profiles

	Difference in mean relative abundance ± standard error of mean				
Phylum	−80 °C vs 4 °C fridge	−80 °C vs OMNIgene.GUT	−80 °C vs RNA later	−80 °C vs TE buffer	−80 °C vs RT
Firmicutes	2.4 ± 1.4	3.6 ± 0.8	7.8 ± 1.6	12.7 ± 1.2	10.5 ± 0.9
Bacteroidetes	2.0 ± 0.5	6.9 ± 1.1	13.6 ± 3.2	12.2 ± 2.4	3.6 ± 0.2
Actinobacteria	1.7 ± 0.3	4.1 ± 1.7	7.0 ± 2.4	9.2 ± 1.7	5.6 ± 2.5
Proteobacteria	0.04 ± 0.02	1.0 ± 0.4	1.3 ± 0.4	10.0 ± 2.3	1.8 ± 1.8

Vliv skladovacích podmínek na bakteriální složení vzorku

Figure 3: Clustering of samples due to storage conditions by PCoA, based on Bray-Curtis similarity distance.

From: Sample storage conditions significantly influence faecal microbiome profiles

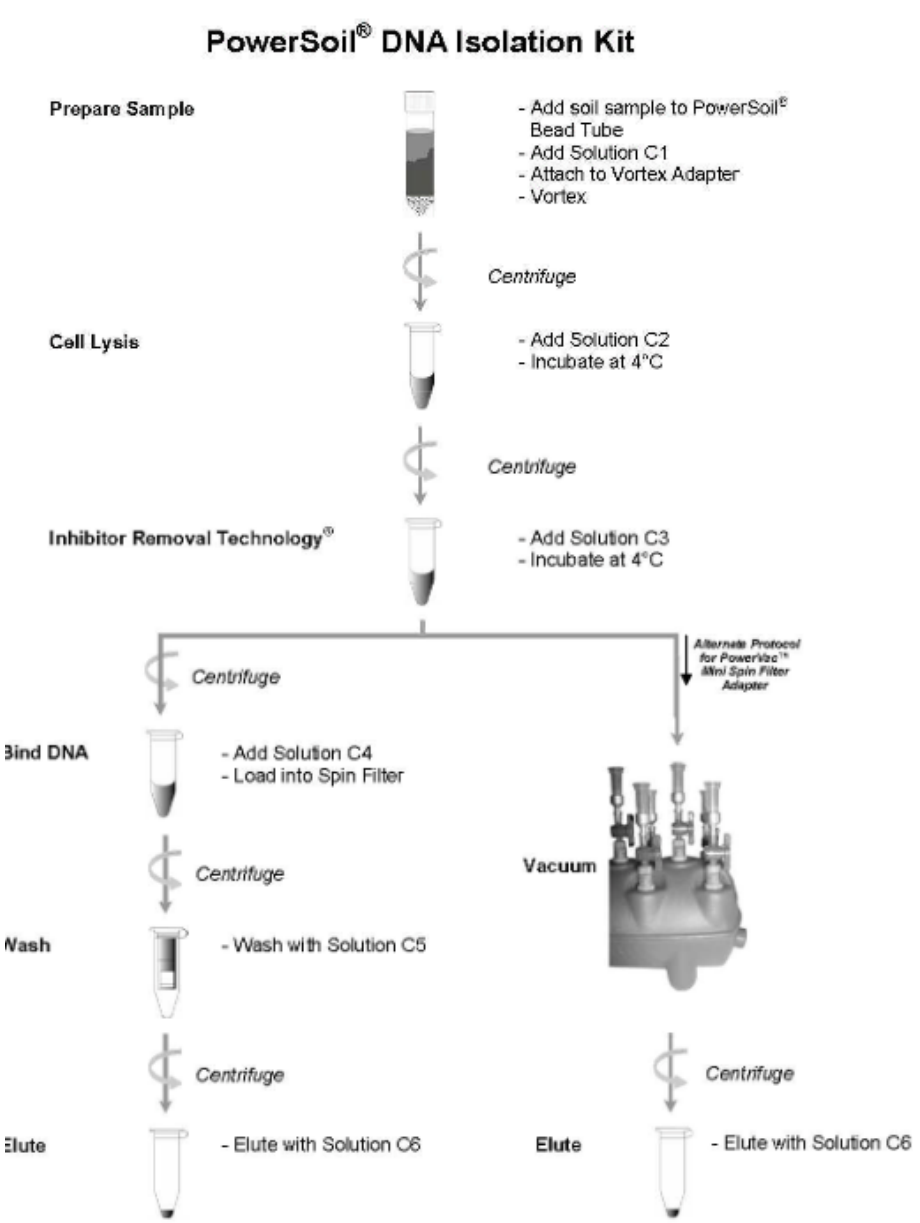


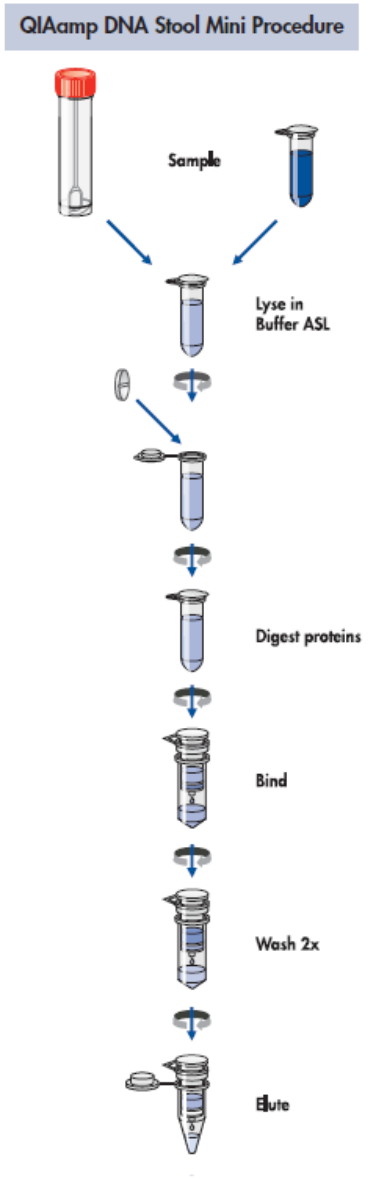
The first two principal coordinates are plotted on the x- and y-axes, respectively (representing 57.3% of the total variation). Faecal collections sampled at three different time points are represented by circle (●) for collection 1, triangle (▲) for collection 2, and square (■) for collection 3. Storage conditions are indicated by colour.

Izolace DNA

- Nejčastěji komerčními kity – přímo dle typu vzorku
- 2 přístupy
  - Lyze buněk enzymaticky
  - Lyze buněk enzymaticky i mechanicky pomocí beat beateru, homogenizátoru →ke vzorku se přidají kuličky z různých materiálů dle výrobce, tento krok lze přidat i u pouze enzymatických kitů
- Doporučuji přidat krok s RNázou A, pokud není součástí postupu

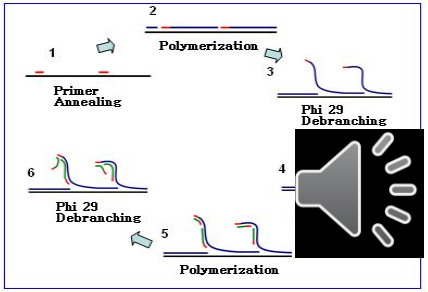
Izolace


PowerSoil® DNA Isolation Kit
Prepare Sample
- Add soil sample to PowerSoil® Bead Tube
- Add Solution C1
- Attach to Vortex Adapter
- Vortex
Centrifuge
Cell Lysis
- Add Solution C2
- Incubate at 4°C
Centrifuge
Inhibitor Removal Technology®
- Add Solution C3
- Incubate at 4°C
Centrifuge
Alternate Protocol for PowerVac™ Mini Spin Filter Adapter
Bind DNA
- Add Solution C4
- Load into Spin Filter
Centrifuge
Vacuum
Wash
- Wash with Solution C5
Centrifuge
Centrifuge
Elute
- Elute with Solution C6
Elute
- Elute with Solution C6


QIAamp DNA Stool Mini Procedure
Sample
Lyse in Buffer ASL
Digest proteins
Bind
Wash 2x
Elute
Fully automatable on the QIAcube

Cíle izolace

- Získat co nejméně degradovanou DNA
  - Degradovaná DNA pro sekvenaci genu 16S rRNA není problém, ale pro celometagenomové sekvenování už může být – lze řešit vyřezáním z gelu
- Získat DNA bez inhibitorů (každý kit obsahuje jinou techniku pro odstraňování inhibitorů)
- Získat co největší množství DNA (opět více důležité pro celometagenomové sekvenování)
  - Pokud není DNA dost – MDA (WGA)



Kontaminace izolačních kitů

Problém zejména u diagnostiky, tam kde je málo vstupní DNA a kontaminace se projeví

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RESEARCH ARTICLE Open Access

Reagent and laboratory contamination can critically impact sequence-based microbiome analyses

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Abstract

Background: The study of microbial communities has been revolutionised in recent years by the widespread adoption of culture independent analytical techniques such as 16S rRNA gene sequencing and metagenomics. One potential confounder of these sequence-based approaches is the presence of contamination in DNA extraction kits and other laboratory reagents.

Results: In this study we demonstrate that contaminating DNA is ubiquitous in commonly used DNA extraction kits and other laboratory reagents, varies greatly in composition between different kits and kit batches, and that this contamination critically impacts results obtained from samples containing a low microbial biomass. Contamination impacts both PCR-based 16S rRNA gene surveys and shotgun metagenomics. We provide an extensive list of potential contaminating genera, and guidelines on how to mitigate the effects of contamination.

Conclusions: These results suggest that caution should be advised when applying sequence-based techniques to the study of microbiota present in low biomass environments. Concurrent sequencing of negative control samples is strongly advised.

Keywords: Contamination, Microbiome, Microbiota, Metagenomics, 16S rRNA

Kontaminace izolačních kitů

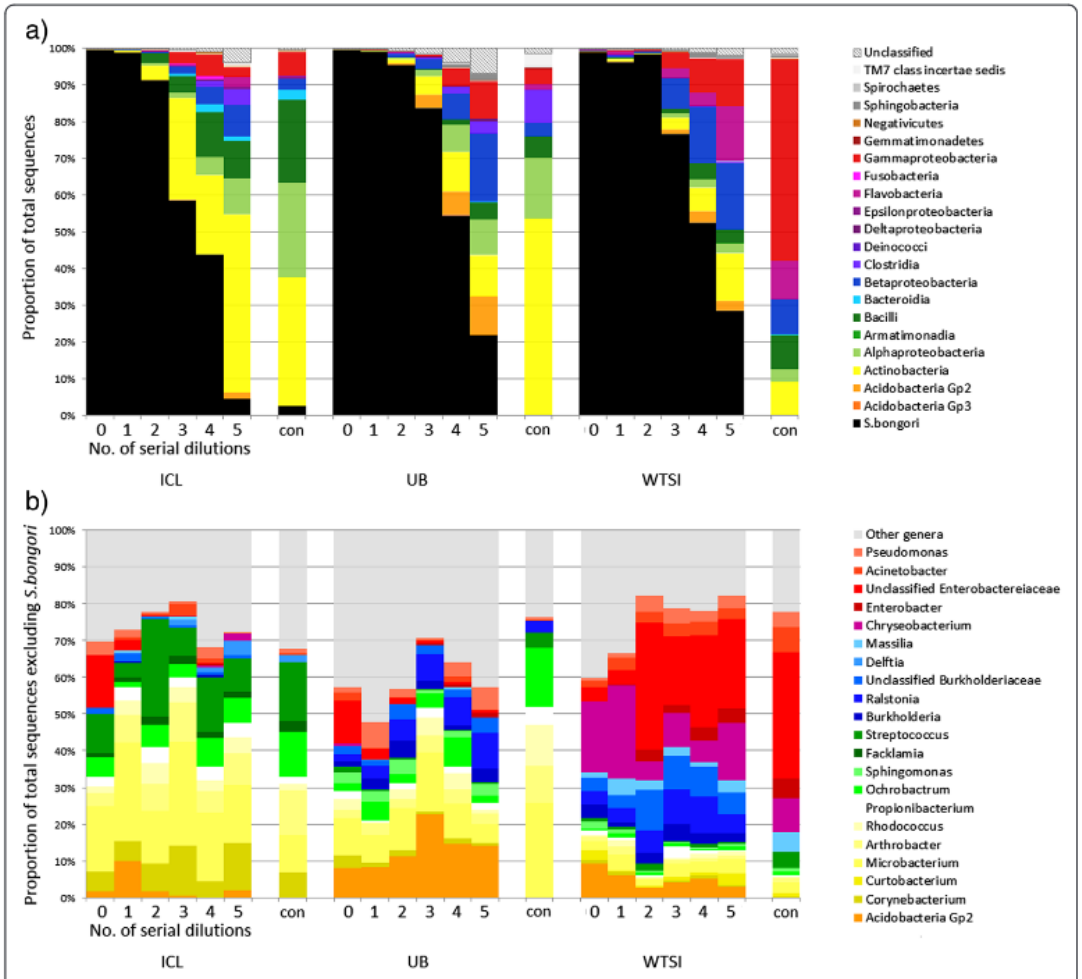

Figure 1 Summary of 16S rRNA gene sequencing taxonomic assignment from ten-fold diluted pure cultures and controls. Undiluted DNA extractions contained approximately 10^8 cells, and controls (annotated in the Figure with 'con') were template-free PCRs. DNA was extracted at ICL, UB and WTSI laboratories and amplified with 40 PCR cycles. Each column represents a single sample; sections **(a)** and **(b)** describe the same samples at different taxonomic levels. **a)** Proportion of *S. bongori* sequence reads in black. The proportional abundance of non-*Salmonella* reads at the Class level is indicated by other colours. As the sample becomes more dilute, the proportion of the sequenced bacterial amplicons from the cultured microorganism decreases and contaminants become more dominant. **b)** Abundance of genera which make up >0.5% of the results from at least one laboratory, excluding *S. bongori*. The profiles of the non-*Salmonella* reads within each laboratory/kit batch are consistent but differ between sites.

Kontaminace izolačních kitů

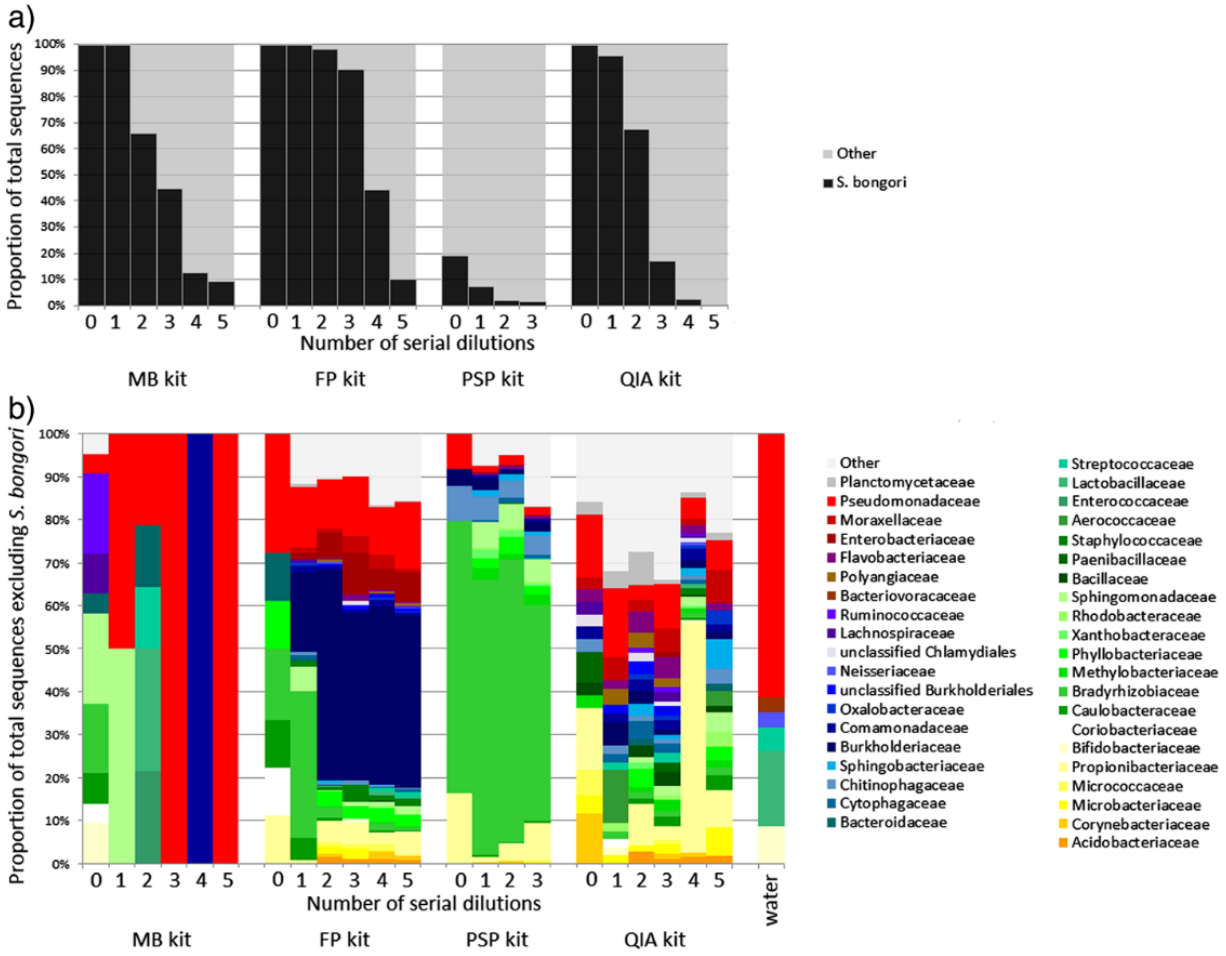

Figure 3 Summary of the metagenomic data for the *S. bongori* ten-fold dilution series (initial undiluted samples contained approximately 10^8 cells), extracted with four different kits. Each column represents a single sample. A sample of ultrapure water, without DNA extraction, was also sequenced (labelled 'water'). **a)** As the starting material becomes more diluted, the proportion of sequenced reads mapping to the *S. bongori* reference genome decreases for all kits and contamination becomes more prominent. **b)** The profile of the non-*Salmonella* reads (grouped by Family, only those comprising >1% of reads from at least one kit are shown) is different for each of the four kits.

Srovnání kitů