Utility of a portable desiccant system for preservation of fecal samples for downstream 16S rRNA amplicon sequencing

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Abstract

While recent advances in culture-independent sequencing approaches have revitalized the field of microbiology, rapid collection and preservation of microbial DNA in samples like feces is critical to avoid degradation of target DNA via nuclease activity and proliferation of aerotolerant microbes. Common laboratory practices to ameliorate such changes rely on prompt freezing of samples or dispersion in nuclease-inhibiting reagents. As many of the microbial enzymes associated with nuclease activity and bacterial proliferation are hydrolases, prompt desiccation of samples offers an attractive alternative to freezing and liquid reagents for field collection of samples in remote areas. Herein, we evaluated the utility of a portable desiccant chamber with a rechargeable cartridge, for preservation of equine fecal samples for downstream microbial profiling via 16S rRNA amplicon sequencing. Controls included matched samples promptly frozen at −80°C or left at room temperature for an equivalent period of time. While samples held at room temperature showed a significant reduction in richness and proliferation of several facultative anaerobes, desiccated samples showed minimal change from promptly frozen samples, with the exception of increased abundance of *Acinetobacter* spp. in desiccated samples relative to frozen samples. The data support the utility of portable desiccant chambers for the preservation of microbial field samples intended for downstream sequencing approaches.

Keywords

16S rRNA gene; Desiccation; Horse; Microbiota; Preservation

1. Introduction

During the last 15 years, the field of microbiology has experienced a renaissance, owing to the development of culture-independent molecular methods of characterizing

microbial communities. One of the most readily available methods exploits characteristics of the partially conserved bacterial and archaeal 16S rRNA gene, encoding the small ribosomal subunit, to identify and quantify multiple members of highly complex microbial populations. Such methods have been widely employed to investigate diverse bacterial ecosystems ranging from the human gut microbiota (GM) to extremophile communities in deep-sea vents. These methods have also provided highly compelling data generated from environmental and host-associated samples collected from sites necessitating field preservation of samples. Notably, host-associated GM samples are dominated by obligately anaerobic organisms, many of which will not survive exposure to ambient oxygen. Furthermore, fecal material often contains considerable nuclease activity such that any DNA released form dead or lysed bacteria will begin to degrade rather rapidly. To obviate these factors capable of skewing downstream results, researchers typically either place samples directly into a lysis buffer containing nuclease inhibitors (requiring the transport of bulky liquid materials for appropriate sample preservation) or freeze the samples promptly following collection.

DNA is degraded by several classes of endo- and exonuclease, with varying affinities for single- or double-stranded nucleic acids and a wide range of sequence specificities. That said, the International Union of Biochemistry and Molecular Biology classifies the vast majority of these enzymes as hydrolases (EC 3.1), requiring the availability of water for the hydrolytic cleavage of the phosphodiester bonds between nucleic acid monomers. Thus, rapid desiccation of samples represents an attractive means of inhibiting degradation of microbial DNA following field collection of anaerobic samples intended for next-generation sequencing. The following study, performed using fecal samples collected from 16 horses of various ages and breeds, was designed to assess the preservation of microbial community structure in rapidly desiccated samples. Freshly evacuated samples were divided into three portions and either promptly placed in a −80°C freezer (the current gold standard for preservation of microbial DNA), or placed in the DriBank desiccant system for 24 or 72 h at room temperature and then transferred to a −80°C freezer. To serve as a control and determine the degree of change associated with delayed preservation of DNA in ambient conditions, the third portion was left at room temperature for 24 to 72 h, and then placed in a −80°C freezer. DNA was then extracted from matched samples simultaneously to obviate any potential effects of extraction date, and used as template to generate 16S rRNA amplicon libraries which were subsequently sequenced using the Illumina MiSeq platform.

2. Results

2.1. Variable reduction in sample mass following desiccation

Portions of fecal material placed in the desiccant chamber were weighed prior to and following desiccation over increasing periods of time to determine the amount of water weight removed. The desiccant material is visible and will undergo a change in color from light blue to light gray as its capacity to remove moisture is reached. Notably, while the desiccant cartridge at 24 h appeared between 50% and 100% exhausted based on subjective comparison to a card provided by the manufacturer, those cartridges containing equivalent masses of fecal material for 30 or 72 h were universally 100% exhausted, based on the color

change. Starting with a mean (± SEM) mass of 302 (± 16.8) mg starting material, samples were reduced to 49.5% (± 9.0%), 30.3% (± 4.8%), and 10.7% (± 1.6%) of their starting weight following 24, 30, or 72 h in the desiccant chamber (Supplementary Fig. 1).

Comparison via ANOVA detected a significant time-dependent increase in the water mass removed ($p = 0.019$; F = 5.499).

2.2. Microbial community structure largely preserved in desiccated samples

Following extraction of DNA, 16S rRNA gene amplicon libraries were generated using single-indexed primers, and libraries were sequenced on the Illumina MiSeq platform. Annotated to the level of family, stark differences between samples kept at −80°C and those remaining at room temperature (RT) are readily apparent on visual inspection (Fig. 1).

Samples desiccated using the DriBank system showed subtle differences from the samples kept frozen until processing but there was good overall agreement between matched samples in these two groups. One exception to this was the apparent proliferation in four of the desiccated samples of bacteria in the genus *Acinetobacter*. This same genus represents the dominant family observed in the RT samples, indicating a rapid doubling time and aerotolerance.

2.3. Richness and α-diversity of desiccated samples matches that of frozen samples

While the overall community structure appeared subjectively similar, it is possible that rare taxa not readily evident in the stacked bar charts were lost during the desiccation process. To test for such an event, we compared richness (i.e., the number of operational taxonomic units detected), and α-diversity (i.e., the richness and evenness of distribution) using two different metrics. To account for potential effects of differential coverage on these metrics, data were first subsampled to a uniform coverage of 65,970 reads per sample. Regarding the detected richness in samples frozen or desiccated prior to DNA extraction, while the RT samples demonstrated a significant ($p < 0.001$) decrease relative to the frozen and DriBank samples, there was no difference in richness between matched frozen and DriBank samples ($p = 0.942$) (Fig. 2A). Similarly, comparison of the Shannon and Simpson α-diversity indices revealed very similar results with no differences between frozen and DriBank samples, and a significant reduction relative to both groups in samples left at RT (Fig. 2B and C).

2.4. No differences in β-diversity between desiccated and promptly frozen samples

As comparisons of richness and α-diversity are based on the number and distribution, but not the identity, of detected taxa, differences in community structure were next visualized and tested using principal coordinate analysis (PCoA) and PERMANOVA respectively. As PCoA and PERMANOVA are predicated on inter-sample distances and those distances can be determined multiple different ways, we replicated all comparisons using both the unweighted Jaccard similarity index and weighted Bray-Curtis similarity index. Considering first the agreement of matched samples based on the Jaccard index, PCoA revealed close agreement in the community structure of frozen and desiccated samples, and a significant shift in the RT samples relative to frozen samples ($p = 0.0009$; F = 2.138) (Fig. 3A). No difference was detected between frozen and DriBank samples based on PERMANOVA

using the Jaccard index ($p = 0.974$; F = 0.546). When the same comparisons were performed using the Bray–Curtis similarity index, the frozen and DriBank samples again demonstrated almost complete overlap upon ordination, while samples kept at RT were substantially shifted along the first principal coordinate (Fig. 3B). Similarly, PERMANOVA detected a significant difference between frozen and RT samples ($p = 0.0001$; F = 8.395) and no difference between frozen and desiccated samples ($p = 0.385$; F = 1.078). Thus, whether inter-sample similarity was determined based on the shared presence or absence of taxa (i.e., the Jaccard index) or the agreement between samples in the relative abundance of shared taxa (i.e., the Bray-Curtis index), no significant differences were detected between the frozen and DriBank-preserved samples. As PERMANOVA testing does not account for matched samples, we also calculated the mean intra-sample distance between each treatment, again using both Jaccard and Bray-Curtis indices. In accordance with the PERMANOVA testing, the intra-sample agreement between frozen and DriBank samples was significantly greater than between frozen and RT samples, regardless of the metric used (Supplementary Fig. 2).

A comparison of the ten most abundant taxa in the frozen samples revealed a trend of decreased abundance in samples maintained at RT (Fig. 4A). Statistical testing confirmed a significant main effect of sample treatment for all ten OTUs. Pairwise comparisons of DriBank and RT samples against the gold standard method of sample preservation (i.e., frozen) detected significant decreases in the relative abundance of all 10 OTUs in the RT samples, and no significant difference in the relative abundance of any OTUs in DriBank-preserved samples after correction for multiple testing. Of the remaining OTUs, the ten most abundant taxa in RT samples were dominated by *Acinetobacter* and *Bacillus* spp., several of which were also subjectively increased in the DriBank samples as well (Fig. 4B). However, pairwise comparisons to the frozen samples detected significant increases in nine of ten OTUs in RT samples (*Acidovorax* sp., p = 0.071 in RT samples versus frozen), and no significant differences between DriBank and frozen samples in any of the ten OTUs after correction for multiple testing. Taken collectively, these data suggest that the predominantly anaerobic dominant taxa found in promptly frozen samples are replaced by aerobic (e.g., *Acinetobacter* spp.) and facultatively anaerobic (e.g., *Bacillus* spp.) microbes when samples are left at RT, and that rapid desiccation of samples will largely ameliorate those changes.

3. Discussion

The study described here was designed to test the utility of a light weight desiccant system for preservation of fecal samples for downstream microbial profiling via the popular 16S rRNA amplicon sequencing approach. For use as mock samples, we selected equine feces based on the high richness relative to that of common laboratory rodents and companion animals (i.e., cats and dogs). Our experimental treatment was meant to mimic collection of a field sample and use of the device for between 24 and 72 h before the sample could be placed in a freezer. For comparison, our positive control was immediate placement of the sample at −80°C; the negative control, demonstrating the need for preservation of some sort, was room temperature storage for the same duration as its matched sample in the desiccant device. It should be noted that the ambient temperatures at which the desiccant and RT

samples were stored was in a climate-controlled research facility. Results of the negative control samples could vary widely depending on the climate in which samples are collected.

While a handful of studies have compared alternative methods of preservations of fecal samples for downstream sequencing or other molecular methods, those studies either compared liquid reagents such as RNAlater or ethanol (which are less than ideal for collection of field samples) (Vlckova et al., 2012; Song et al., 2016; Hale et al., 2015; Al et al., 2018), or Whatman FTA cards. The latter produced disparate results depending on the sample type and require the sample to be in a liquid suspension. The DriBank system requires no liquids at all and can be used with relatively dry, fiber-rich samples such as equine feces.

Supporting its utility in collection of field fecal samples for similar downstream assays, matched samples preserved in the DriBank device showed only minor compositional change from promptly frozen samples, while samples left at room temperature for comparable periods of time experienced substantial overgrowth of several aerobic or facultative taxa and a significant decrease in richness. The amelioration of both of these changes in desiccated samples reflects the common use of hydrolases in nuclease function and bacterial cell growth (Lee and Huang, 2013; Vollmer, 2012). That said, a few of the desiccated samples showed an apparent proliferation of taxa identified as *Acinetobacter* spp. relative to the matched frozen samples, perhaps a consequence of its rapid doubling time (Henry et al., 2012) or differential moisture content in the original sample. The pre- and post-desiccation sample weights were compared between those samples in which Acinetobacter did or did not proliferate and no differences were detected. That said, future studies are needed to determine the maximal moisture capacity of the desiccant cartridges and effects of variable moisture content on efficacy. Notably, one study examining the effects of time post-defecation at ambient temperature on the composition of equine feces also detected significant increases in the relative abundance of microbes in the families *Bacillaceae* and *Planococcaceae* (as well as *Enterococcaceae*), and a trend ($p = 0.055$) toward increased abundance of family Moraxellaceae (Beckers et al., 2017). The close agreement of the current room temperature samples with those results (generated in Southern Louisiana) suggests that the changes observed with sub-optimal sample handling of equine feces are relatively conserved across geographical regions, and that the DriBank instrument was capable of abrogating the majority of those changes.

4. Conclusions

Collectively, the data presented here suggest that the rapid desiccation of samples using the DriBank device was, with minor exception, effective at preserving the overall microbial composition of rich polymicrobial samples. Rapid desiccation of fecal samples largely abrogated the loss of taxa via nuclease activity as well as the opportunistic growth of facultative anaerobes. These findings support the use of such a system for collection of fecal samples in environments in which rapid freezing or transport of liquid reagents is problematic.

5. Materials and methods

5.1. Sample collection

All samples were freely evacuated from adult horses of various ages and breeds, staying in the University of Missouri Veterinary Health Center, large animal clinic. Samples had been evacuated no > 60 min prior to collection and were promptly placed in sealed polypropylene containers and transported immediately to the lab.

5.2. Sample processing and desiccation

First, approximately 300 mg of fecal material was removed from the original sample, weighed, gently pulled apart, and placed in the inverted cap of a sterile 50 mL conical tube labeled with the sample ID. Four such samples at a time were then sealed in the DriBank device with a new or freshly recharged desiccant cartridge, and allowed to remain on the laboratory bench-top (approximately 20°C) for 24, 30, or 72 h. Following desiccation, samples were re-weighed and then transferred to a sterile 50 mL conical tube and placed in a −80°C freezer. A second portion of each sample (approximately 0.5 to 1 g) was gently pulled apart, placed in a sterile 50 mL conical tube, and placed in a −80°C freezer. The remainder of the sample was gently pulled apart, left in a sealed polypropylene container on the bench-top adjacent to the DriBank device for the same duration as their matched samples, and then transferred to −80°C.

5.3. DNA extraction

DNA was extracted using the entirety of the sample placed in the DriBank device, or roughly 250 mg of sample from the frozen and RT portions, using PowerFecal DNA Isolation kits (Qiagen) according to the manufacturer's instructions. The only adaptation was the replacement of the recommended vortex and adaptor with a Qiagen TissueLyser II (3 min at 30 Hz) for initial sample homogenization. Following extraction, double-stranded DNA was quantified via fluorometry (Qubit 2.0, Life Technologies) using the quant-iT BR dsDNA reagent kits (Invitrogen).

5.4. 16S rRNA amplicon library preparation and sequencing

Library preparation was performed at the University of Missouri DNA Core as previously described (Ericsson et al., 2015). Briefly, the V4 region of the 16S rRNA gene was amplified using previously published primers with single-indexed universal primers (U515F/ 806R) flanked by Illumina adapter sequences (Walters et al., 2011; Caporaso et al., 2011). Amplified products from each reaction were then pooled and purified using Axygen AxyPrep MagPCR Clean-up beads. The final purified amplicon pool was then evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified via fluorometry as before, and diluted according to the standard protocol for sequencing on the Illumina MiSeq.

5.5. Informatics

All assembly, binning, and annotation of DNA sequences was performed at the University of Missouri Informatics Research Core facility as previously described (Ericsson et al., 2015). Briefly, contiguous DNA was assembled using FLASH software and filtered if base

quality was < 31. Qiime v1.9 software (Kuczynski et al., 2011) was used to perform de novo and reference-based chimera detection and removal, and taxonomy was assigned to operational taxonomic units using BLAST (Altschul et al., 1997) against the SILVA database (Pruesse et al., 2007; Quast et al., 2013). Following annotation, data were sub-sampled to a uniform coverage of 65,970 reads per sample using the beta_diversity_through_plots.py package available at http://qiime.org/scripts/beta_diversity_through_plots.html. Metrics of richness and α-diversity were determined using PAST 3.16 (Hammer, 2017). Sequence data have been uploaded to the NCBI Sequence Read Archive (SRA) under accession number PRJNA423231.

5.6. Statistical analysis

Testing for differences in richness, α-diversity indices, and the relative abundance of select OTUs was performed using a one-way repeated measures (RM) analysis of variance (ANOVA) or Friedman RM ANOVA on ranks for normally and non-normally distributed data respectively, with post hoc pairwise comparisons according to Dunnett's method, implemented in SigmaPlot 13.0. Differences in β-diversity were tested using one-way permutational multivariate ANOVA (PERMANOVA) using Past 3.16.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations:

OTU operational taxonomic unit

PCoA principal coordinate analysis

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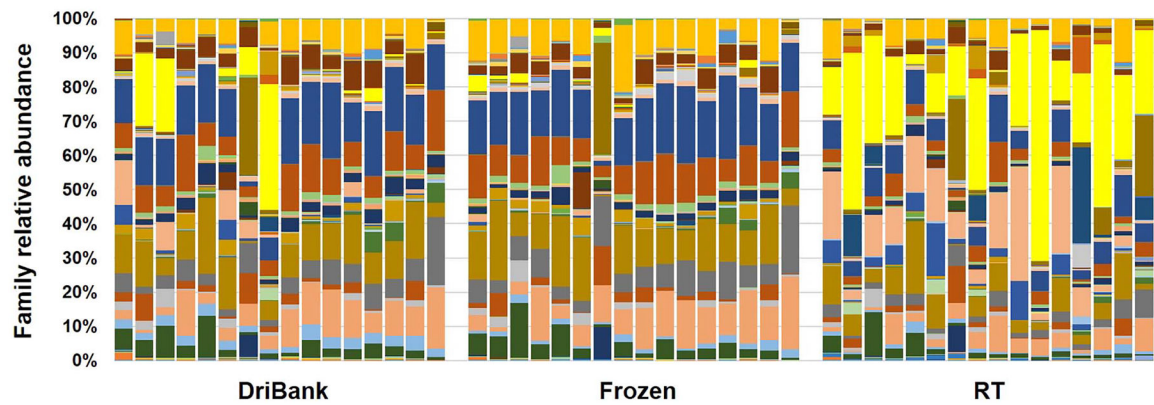
Fig. 1.
Stacked bar chart showing microbial communities, annotated to the taxonomic level of family, of 16 freshly evacuated equine fecal samples desiccated in the DriBank device, maintained at −80°C until processing (Frozen), or kept at room temperature for 24 h (RT). Genus *Acinetobacter* (in family *Moraxellaceae*) shown in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
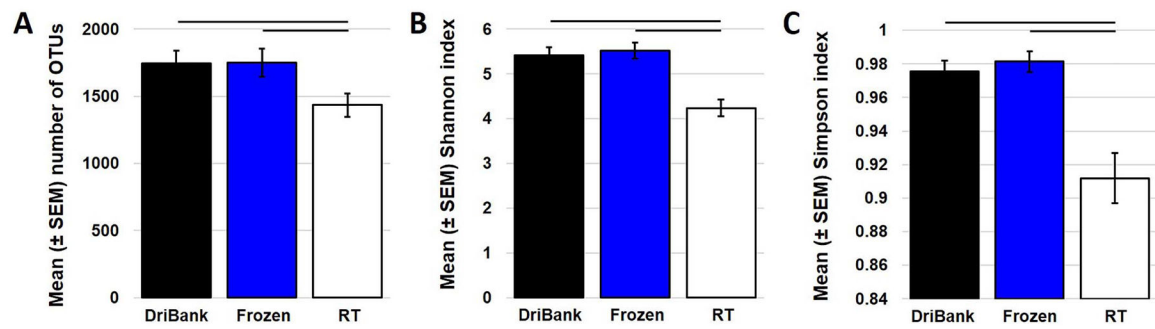
Fig. 2.
Bar charts showing mean (± SEM) number of operational taxonomic units (OTUs) (A), Shannon diversity index (B), and Simpson diversity index (C) in 16 freshly evacuated equine fecal samples desiccated in the DriBank device, maintained at −80°C until processing (Frozen), or kept at room temperature for 24 h (RT). Bars indicate significant differences between groups based on one-way repeated measures ANOVA.
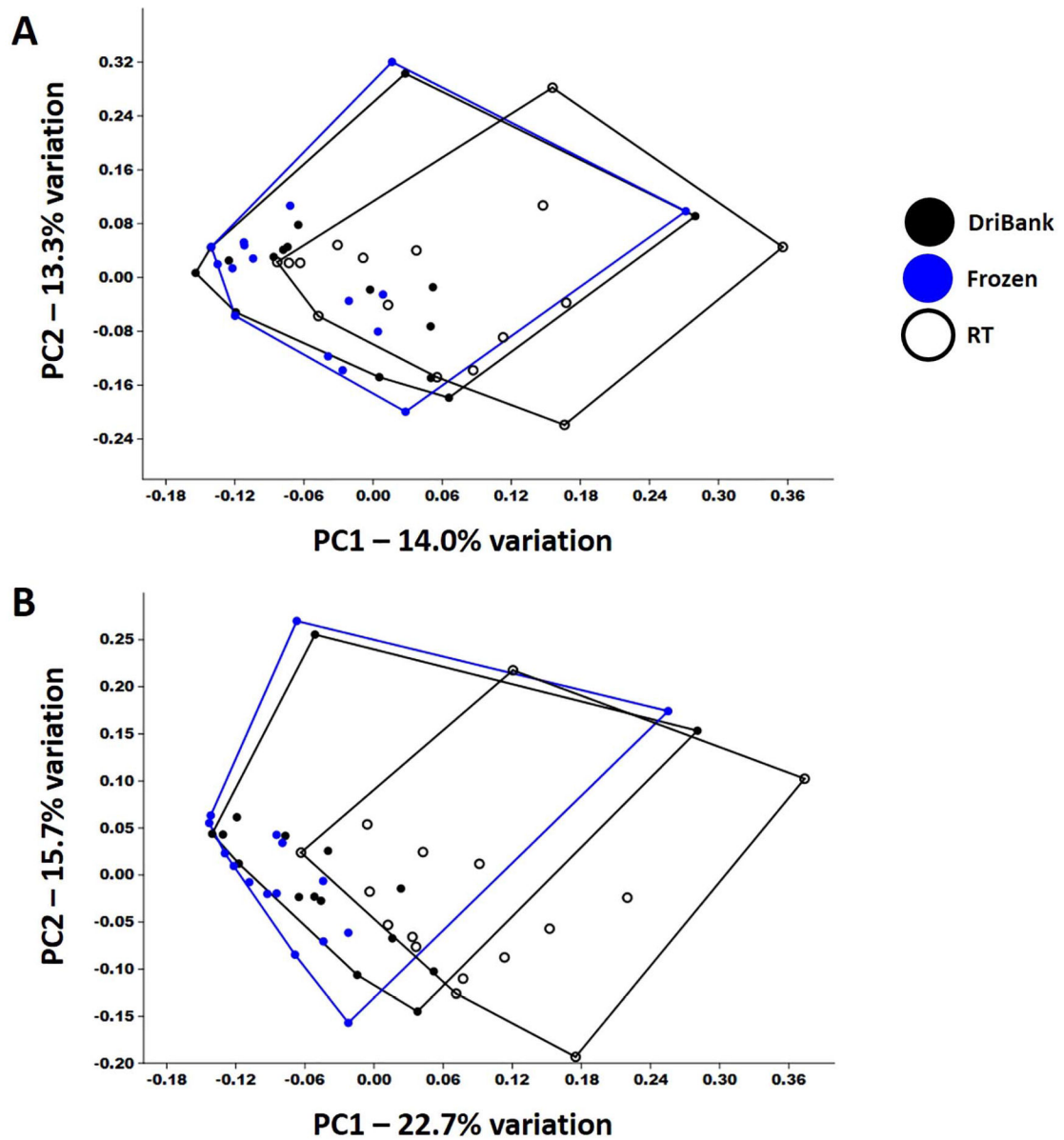
Fig. 3.
Principal coordinate analyses based on Jaccard (A) and Bray-Curtis (B) distances, showing the relationship between community structure of promptly frozen samples and matched samples desiccated using the DriBank system or left at room temperature (RT) for 24 to 72 h.
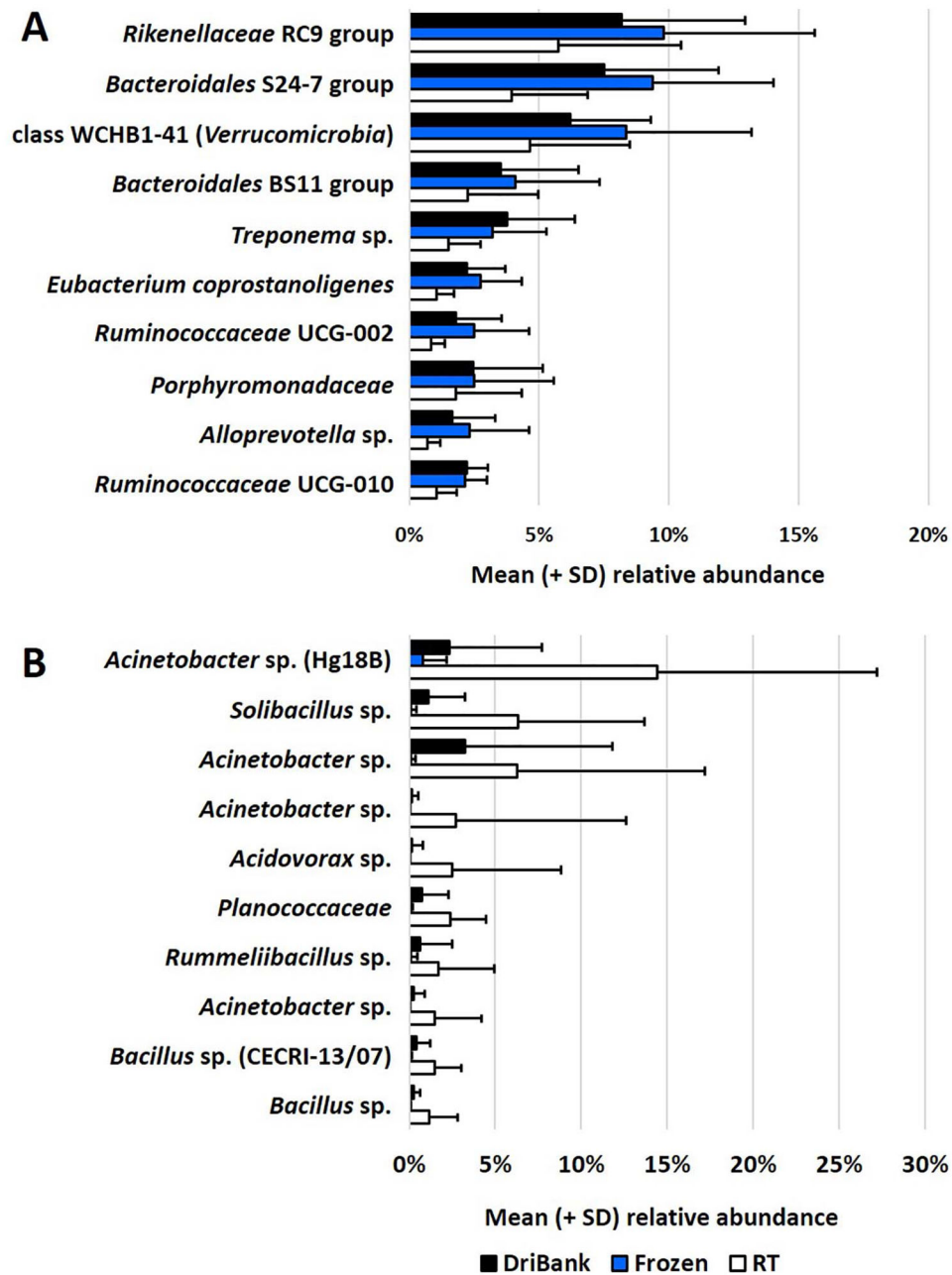
Fig. 4.
Bar chart showing mean relative abundance in all treatment groups of the ten most abundant OTUs in promptly frozen samples (A), and the ten most abundant OTUs of the remaining taxa in samples kept at room temperature (RT) (B).