5-2020

The Effects of Storage Temperature on the Bovine Nasal Microbiome

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The Effects of Storage Temperature on the Bovine Nasal Microbiome

An Honors Thesis submitted in partial fulfillment of the requirements of Honors Studies in Animal Science

by Hunter Usdrowski

Spring 2020

Animal Science
Dale Bumpers College of Agricultural, Food and Life Sciences
University of Arkansas

Acknowledgements

I offer a special thanks to both Dr. Julie Stenken's and Dr. Jianchao Zhao's lab groups for their immeasurable guidance, wisdom and immense support in making this research possible. I would like to especially thank Jianmin Chai, a member of Dr. Zhao's group, for his time-consuming efforts in assisting me throughout my time and research as an undergraduate. Another special thanks to a member of Dr. Stenken's lab and a fellow colleague, Srusti Maddala, for her unwavering motivation and support. This research was supported by an Honors College grant.

Contents

Abstract

Bovine respiratory disease (BRD) is one of the largest economic burdens facing United States beef producer's modern day. The complexity in the onset and development of this multifactorial disease necessitates further insights into its functions to alleviate the tremendous consequences it generates for producers. The respiratory microbiome and their metabolomics play an important role to maintain health and predict BRD. However, due to low biomass, new technology is needed to increase the microbial concentration for omics (e.g. metatranscriptomics) research. In this study, a novel self-enrichment storage technique on the bovine nasal microbiome was performed and compared to a traditional nasal sample storage method to further develop microbial communities. To assess these methods, two nasal swab samples from 2 cohorts (10 calves each) at two different locations were collected at two different timepoints. Subsequently, one set of samples was subjected to the novel technique (room temperature culture for 7 days in Amies buffer then stored at -80C, RT), while the other set followed traditional storage protocol (stored into -80C directly, UL) as a control. The nasal microbiome was then characterized using 16S rRNA sequencing of the V4 region. The RT storage technique was characterized by a significant decrease in microbial diversity and richness when compared to UL storage samples for both locations and timepoints ($p<0.05$). Furthermore, RT samples showed distinct clustering from UL samples for both locations and timepoints when measured by both Jaccard and Bray-Curtis distances. Community structure between the storage techniques was also assessed at the genus level, characterized by a reduction in common airway genera such as *Moraxella* and *Pasteurellaceae* and an increase in common genera such as *Enterococcus* and *Pseudomonas* when the RT storage technique was compared to traditional UL storage. Random forest was

found as an accurate model used to biomark and rank the most influential species differentiating the storage techniques. OTUs associated with BRD pathogens were identified as UL biomarkers, such as OTU53-*Mycoplasma*, OTU9-*Moraxella* and OTU35-*Pasteurellaceae*, while OTU1-*Enterococcus* and Otu18-*Streptococcus* were consistently observed to increase in RT, consistent with the corresponding genus shift. Finally, Procrustes analysis using Jaccard distance was used to determine the consistency in RT storage influence on the nasal microbiome among samples compared to UL storage. Consistent trend among samples was observed when comparing the RT storage technique to UL storage across both locations and timepoints. In summary, the novel self-enrichment room temperature storage technique was found to enrich specific microbiota but ultimately shifted the microbial structure of the "normal" respiratory community. Thus, future improvement and investigation into the novel self-enrichment technique is necessary to expand its uses for further analysis of the microbiomes function in the onset and development of BRD.

Introduction

The United States is the largest producer of beef in the world, holding an inventory of approximately 31.7 million beef cows as of January 31, 2018 (Drouillard, 2018). One of the largest economic challenges associated with producing such an immense quantity of beef is the presence of Bovine Respiratory Disease, generated by a multifactorial complex of predisposed, environmental related stress and host tolerance, as well as varying respiratory pathogens. Incidence of disease reported by the National Animal Health Monitoring System (NAHMS) indicates approximately 14.4% of cattle that reach feedlots develop BRD, accounting for 70-80% of morbidity and 40-50% of mortality in this production phase (Edwards, 2010). The combined costs of initial and subsequent treatment, decreased weight gain efficiency and mortality from BRD result in over 800 million dollars in economic losses annually, creating significant financial strain on producers and consumers surrounding the industry (Chirase & Greene, 2001).

Better understanding the onset of BRD, specifically temporal microbiome dynamics, can provide researchers and producers with more cost effective and successful applications. Additionally, technologies such as the development of next-generation sequencing techniques have allowed researchers to gain a more wholistic portrayal of microbiota influences within the bovine airway, providing an enhanced characterization of the bovine nasal microbiome. Traditionally, several opportunistic bacterial pathogens of BRD such as *Pasteurella multocida*, *Mycoplasma bovis*, *Mannheimia haemolytica*, and *Histophilus somni* have been the primary focus of BRD studies. However, each of these pathogens are known to exist in both “healthy” and morbid animals (Holman, Timsit, & Alexander, 2015). Therefore, more accurately characterizing the dynamics of the nasal microbiome and metatranscriptome, including the mentioned opportunistic pathogens

and all other uncultured bacteria, is crucial for a larger understanding of the potential pathogens involved in developing BRD.

Sampling microbial populations from the bovine nasal cavity has previously been performed by swabbing the mid-nare mucosal lining of the airway membrane, and subsequently moving the samples stored in a non-nutritive transport medium to ultra-low temperature storage at -80°C until nucleic acid extraction (McDaneld, Kuehn, & Keele, 2018). This process remains adequate for the analysis of the microbiota, however, does not always provide enough quantity of total RNA for more in-depth study of the microbiome functions, e.g. the metatranscriptome. A potential method to overcome this problem is storing nasal swab samples at room temperature (RT) to increase the cell density of the culture, thus raising the overall total RNA output. Utilizing sampled mucous contained within the matrix of the swab, population biases from standard growth media sources may be reduced while achieving adequate total RNA. Ultimately, variation of storage temperature may provide a self-enrichment method for microbials to increase cell density inside nasal swab cultures, allowing the potential for necessary downstream metatranscriptomic data, providing further insight in to bacterial-bacterial and bacterial-host interactions throughout the development of BRD.

Statistical and ecological analysis of samples alpha and beta diversities to compare the effects hypothesized storage protocol has on the bovine nasal microbiota will have future implications regarding more in-depth microbiome analysis. Advances in this type of analysis have previously helped provide others with the knowledge to develop alternative treatment therapies, such as the use of probiotics, in preventing and treating BRD (Amat, Timsit, Baines, Yanke, & Alexander, 2019). Furthermore, a significant reduction in overall antibiotic usage that contributes to antibiotic

resistant bacteria, the development of higher and more consistent feed efficiencies, and less decreased morbidity and mortality in feedlots could result from this research.

Methods

Ethics Statement

Approval was granted to conduct the following experimental procedures and animal husbandry practices utilizing beef cattle by the University of Arkansas Institutional Animal Care and Use Committee (Protocol # 19071).

Calf Metadata

Two cohorts of 10 calves each were used for collecting nasal swab samples, one cohort located in Savoy, Arkansas (AR) and the other in Stillwater, Oklahoma (OK). The straight-line distance between sampling sites was approximately 150 miles. Samples at both sites were collected at two timepoints separated by 28 days. AR calves received clostridial (Covexin 8) and respiratory complex (Bovi-Shield Gold One) vaccines one month prior to the first sampling timepoint. Subsequent to the first sampling timepoint, AR calves were turned out to pasture and were fed soy hull pellets and offered free choice mineral. Feed was offered at 0.5% of the average weight of the entire group (85 calves). OK calves received a clostridial (Vision 7 Spur) vaccine 3 months prior to the first sampling timepoint. 84 days pre-sampling of the first timepoint, OK calves were placed on a receiving diet measured in % DM in diet as follows: corn, rolled at 15%, sweet bran at 51.36%, B-340 pelleted supplement at 5.20% and prairie hay at 28.44%. All calves at both sites remained healthy, e.g. none were treated for disease, over the course of the study.

Sample Acquisition

Sampling was conducted across two timepoints separated by 30 days. Two Puritan Opti-Swabs (Puritan Medical Products Co. LLC Guilford, Maine, USA) were revolved inside the mid-nare region of calves' right nostrils until swabs were fully coated in mucous, noted by visual inspection. Swabs were then aseptically transferred in to separate liquid Amies buffer transport collection tubes containing 2mL of liquid Amies buffer and were subsequently stored at either immediate 80°C or 7 days at RT with subsequent -80°C storage conditions.

Storage Protocol

RT samples were vortexed upon arrival to the laboratory until full mucous disruption from the swab was observed. Ultra-low temperature samples were directly placed in to the -80°C freezer. Visual inspection of RT samples was conducted every 24 hours until the 7^{th} day, in which samples were subsequently stored at UL temperature until extraction. Samples were labelled corresponding to their subjected treatment **(Figure 1)**.

DNA Extraction and Next-generation sequencing

DNA was extracted from all nasal swab samples using a DNeasy PowerLyzer PowerSoil kit (Qiagen Inc, Germantown, MD) and a negative control swab was implemented to exclude potential contamination. Next-generation sequencing was performed on an Illumina MiSeq sequencer contained in the Biomass Research laboratory within the University of Arkansas's Division of Agriculture. Amplification of the V4 region of the 16S rRNA gene and its sequence on an Illumina MiSeq 2 x 250 platform was sequentially conducted. A sequencing library specifically targeting the V4 region of 16S rRNA was then developed using DNA from each sample, following a previous report (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Amplification of individual DNA samples and a negative water control with dual-index primers were added by

PCR, along with the amplification of amplicons using a SequalPrep™ Normalization kit (Life Technology), in correspondence to the manufacturer's recommendation. PCR amplicons from each sample possessed specific barcode sequences to differentiate identification from one another in the pooled library. A 5uL aliquot of each normalized sample was taken to combine and generate one pooled library for further assays. Library concentration and exact product size was measured using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) through a quantitative PCR (qPCR, Eppendorf, Westbury, NY, USA) assay and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA), respectively. The pooled library was consequently diluted to 4nM, pre-sequencing.

The MiSeq run included described nasal samples, 1 negative control for sequencing, 1 mock community and 2 liberal controls (samples with increased DNA concentration that may be divided into many aliquots) for inter-run variations.

Bioinformatics

Analysis of Next-generation sequencing data was performed using the software mothur v.1.39.1 following the MiSeq SOP (http://www.mothur.org/wiki/MiSeq_SOP) (Schloss et al., 2009). Raw sequences were assembled, and chimeras were removed by the algorithm VSEARCH. Sequences were aligned using the SILVA reference database (full-length sequences and taxonomy references release 132, http://www.arb-silva.de/) (Pruesse et al., 2007). Sequences were then arranged into operational taxonomic units (OTUs) at the 97% similarity level and a naïve Bayesian classifier against the Ribosomal Database Project classified the OTUs (Wang, Garrity, Tiedje, & Cole, 2007). Finally, random subsampling of sequences to the smallest number of reads (1054) to minimize the effect of sequencing depth on alpha and beta diversity measures was conducted.

Statistical Analysis

Following bioinformatics, a series of statistical analyses were run to find the dissimilarities between RT and -80°C samples collected at the two time points from Arkansas and Oklahoma by estimating the microbial structure, richness and composition of predominant microbiota. Alpha diversities (Shannon Index and number of observed OTUs) were calculated and visualized using the 'ggpubr' package in R (v3.6.0). A Wilcoxon test was performed to detect the differences, observed by pair-wise comparison. Beta diversity distance metrics (Bray-Curtis and Jaccard) were visualized using principle coordinates analysis (PCoA) multidimensional plots in R. Analysis of similarity (ANOSIM) tests were used to detect beta diversity statistical significances.

Procrustes analyses using Jaccard distance were performed to compare the correlation of NMDS configurations by scaling and rotating one to the other. The statistical significance of the correlation between the two configurations was calculated by a permutation procedure (protest function).

Random forest, a machine learning algorithm, was utilized in R 'randomForest' package to identify and rank marker OTUs differentiating the RT and -80°C samples. Plots of variable importance were generated by the mean decreased accuracy (MDA) of features. The top 25 features were chosen as the marker predictors. The 'importance' and 'proximity' parameters were set as 'True' and the 'ntree' count was set to 10000 trees in the model.

Results

Cohort Weights

Calves were weighed at each sampling timepoint for both locations and their corresponding group mean weights were recorded **(Figure 2)**.

Storage Temperature Effects

Alpha diversity of samples was measured by the Shannon index and observed OTUs to reveal the dissimilarities between UL and RT storage treatment. The Shannon diversity index was shown to significantly decrease ($p < 0.05$) when comparing RT storage to UL storage conditions using a Wilcoxon rank sum test across 36 of 40 RT samples **(Figure 3A)**. Additionally, alpha diversity as a function of observed OTUs was also shown to significantly decrease when comparing RT storage to UL storage conditions ($p<0.05$) across all 40 RT samples using a Wilcoxon rank sum test **(Figure 3B)**. Principle coordinates analysis (PCoA) plots for both Jaccard and Bray-Curtis beta diversity metrics were developed to visualize the effects of storage temperature among sample populations. Jaccard-based PCoA revealed distinct clustering of RT samples distinctly separate from UL samples **(Figure 4)**. ANOSIM results were used to test the statistical significance between storage temperature treatments based on Jaccard distance and revealed significant dissimilarities among samples from both Arkansas ($R=0.97$, $p<0.05$) and Oklahoma ($R=1$, $p<0.05$), when comparing RT to UL storage temperature. Bray-Curtis-based PCoA also revealed distinct clustering of RT samples separated from UL samples. Consistently, ANOSIM results based on Bray-Curtis distance was used to reveal statistically significant distances among storage temperature treatments, and revealed that both Arkansas ($R=0.93$, $p<0.05$) and Oklahoma ($R=0.95$, $p<0.05$) location were significantly dissimilar when comparing RT to UL storage temperature.

Community structure for all samples based on relative abundance was compared at the phylum, genus and OTU levels for storage temperature treatment effects. At the phylum level, community structure between UL and RT samples was characterized by *Proteobacteria* (38.07%, 36.11%), *Firmicutes* (22.26%, 39.91%) and *Bacteroidetes* (11.94%, 21.11%), respectively **(Figure 7)**. At the genus level, community structure consistently differentiated between UL and RT samples, as

Moraxella (0.36%), *Pasteurellaceae* (1.37%) and *Gammaproteobacteria* (0.04%) consistently decreased and *Enterococcus* (13.79%), *Pseudomonas* (8.16%) and unclassified *Enterobacteriaceae* (7.59%) consistently increased at RT storage **(Figure 8)**. At the OTU level, OTU9-*Moraxella* (0.34%), OTU11-*Gammaproteobacteria* (0.03%) and unclassified OTU12-*Corynebacterium* (0.61%) consistently decreased while OTU1-*Enterococcus* (13.79%), unclassified OTU2-*Enterobacteriaceae* (7.45%), OTU6-*Macellibacteroides* (3.85%), and OTU8-*Pasteurellaceae* (1.34%) consistently increased in RT samples **(Figure 9)**.

Location Effects

Alpha diversity of samples was measured by the Shannon index and observed OTUs to reveal the dissimilarities between AR and Oklahoma subject's nasal microbiomes. The Shannon Index was shown to be significantly higher ($p<0.05$) for OK compared to AR for timepoint 1, but not timepoint 2, based on the analysis of UL samples **(Figure 3 A)**. Observed OTUs were also compared between locations and were found significantly higher ($p<0.05$) for OK compared to AR at timepoint one, however no statistically significant difference was found across location for timepoint 2 based on UL samples **(Figure 3 B)**. Beta diversity was compared using PCoA plots for both Jaccard and Bray-Curtis distances, and statistical significance using ANOSIM was found across locations for both distances. Jaccard PCoA revealed distinct clustering of AR samples compared to OK samples across both timepoints for RT and UL storage temperature **(Figure 4 A)**. The ANOSIM test was also found significantly different for samples belonging to UL1AR compared to UL1OK ($R=0.95$, $p<0.05$), RT1AR compared to RT1OK ($R= 0.70$, $p<0.05$), UL2AR compared to UL2OK ($R=0.67$, $p<0.05$), and RT2AR compared to RT2OK ($R=0.93$, $p<0.05$). Bray-Curtis PCoA also revealed distinct clustering of AR samples compared to OK samples for both timepoints and storage temperature treatments **(Figure 4 B)**. ANOSIM

was also found significantly different for samples from AR and OK locations, with corresponding significances for UL1AR-UL1OK ($R=0.86$, $p<0.05$), RT1AR-RT2OK ($R=0.62$, $p<0.05$), UL2AR-UL2OK ($R=0.47$, $p<0.05$), and RT2AR-RT2OK ($R=0.88$, $p<0.05$).

Community structure at the phylum level was consistently predominated by *Proteobacteria* (44.79%, 29.39%), *Firmicutes* (34.21%, 27.96%) and *Bacteroidetes* (11.78%, 21.27%) across AR and OK samples, respectively **(Figure 7)**. At the genus level, community structure was significantly different between the two sampling locations with *Moraxella* (15.44%), *Pasteurellaceae* (16.21%) and *Gammaproteobacteria* (10.57%) predominating in UL1AR and UL2AR samples and *Corynebacterium* (17.17%), *Moraxella* (8.48%), and *Prevotella* (6.66%) predominating in UL1OK and UL2OK samples **(Figure 8)**. *OTU*11-*Gammaproteobacteria* (10.50%), *OTU*8-*Pasteurellaceae* (9.67%), and *OTU*9-*Moraxella* (9.50%) compared to *OTU*12-*Corynebacterium* (8.15%), *OTU*15-*Corynebacterium* (8.94%) and *OTU*9-*Moraxella* (6.07%) were found to predominate UL1AR and UL2AR samples and UL1OK and UL2OK samples at the OTU level, respectively **(Figure 9)**.

Longitudinal Impact

Alpha diversity of samples was measured by the Shannon index and observed OTUs to reveal the longitudinal shifts across sampling timepoints for the nasal microbiome. The Shannon index was not found to be statistically significantly different between timepoint 1 and timepoint 2 for storage temperature treatment and location **(Figure 3 A)**. However, the number of observed OTUs was found statistically significantly higher for UL1OK when compared to UL2OK ($p<0.05$), while no other storage temperature treatments or locations were found statistically different across timepoint 1 and timepoint 2 **(Figure 3 B)**. Beta diversity between timepoints was compared using PCoA plots for both Jaccard and Bray-Curtis distances, and statistical inferences using the ANOSIM test

were calculated across both timepoints to estimate the temporal effect on the nasal microbiome. Jaccard and Bray-Curtis PCoA plots did not reveal distinct clustering of UL samples when comparing sampling date **(Figure 4)**. However, the ANOSIM test found UL1AR and UL2AR statistically significantly different ($R=0.17$, $p<0.05$), UL1OK and UL2OK statistically significantly different ($R=0.23$, $p<0.05$), RT1AR and RT2AR statistically significantly different ($R=0.77$, $p<0.05$), and RT1OK and RT2OK statistically significantly different ($R=0.61$, $p<0.05$).

Community structure at the phylum level remained consistent as *Proteobacteria* (51.96%, 55.50%,18.43%, 26.37%), *Firmicutes* (23.51%, 15.73%, 27.03%, 22.78%) and *Bacteroidetes* (7.91%, 11.97%, 16.06%, 11.83%) remained the top three relatively abundant phyla for UL1AR, UL2AR, UL1OK, and UL2OK, respectively **(Figure 7)**. At the genus level UL1AR and UL2AR samples top three abundant genera were both found to be *Moraxella* (15.30%, 15.58%), *Pasteurellaceae* (15.21%, 15.58%) and *Gammaproteobacteria* (10.79%, 10.35%), respectively **(Figure 8)**. At the genus level for OK samples, UL1OK samples top three abundant genera were *Corynebacterium* (19.43%), *Prevotella* (7.60%) and *Mycoplasma* (6.09%) compared to *Corynebacterium* (14.92%), *Moraxella* (14.41%), and *Deinococcus* (9.71%) for UL2OK samples (**Figure 8)**. *OTU*11-*Gammaproteobacteria* (10.67%, 10.33%), *OTU*8-*Pasteurellaceae* (9.64%, 9.69%), and *OTU*9-*Moraxella* (10.65%, 8.34%) *were* found as the top three OTUs for both UL1AR and UL2AR samples b **(Figure 9)**. *OTU*15-*Corynebacterium* (10.50%), *OTU*12-*Corynebacterium* (8.87%) and *OTU*11-*Gammaproteobacteria* (3.97%), were found as the top three OTUs in UL1OK compared to *OTU*9-*Moraxella* (10.74%), *OTU*12-*Corynebacterium* (7.43%) *and OTU*15-*Corynebacterium* (7.39%) for UL2OK **(Figure 9)**.

Treatment Predictors Identified by Machine Learning

Random forest was used to rank the importance of marker bacteria across storage, location and longitudinal effects, respectively **(Table 1)**. First, we determined the pair-wise comparison of treatments model accuracy to be greater than 95%. OTUs associated with the common, known BRD pathogens were consistently identified as UL markers, such as OTU53-*Mycoplasma*, OTU9-*Moraxella* and OTU35-*Pasteurellaceae*. Other OTUs, including OTU1-Enterococcus and Otu18-Streptococcus, were found to consistently increase with RT, directly corresponding to characterized genus shifts. Next, the biomarkers differentiating locations were also identified using a machine leaning algorithm with high, greater than 90%, accuracy. Respiratory microbes such as Otu15-*Corynebacterium* and gut microbes including *Prevotella* (OTU166, OTU38, OTU169) were found to be the most influential in OK samples at both timepoints. Microbes known to be related to BRD, such as OTU53-*Mycoplasma* and Otu35-*Pasteurellaceae*, were classified to distinguish AR samples for timepoint one. Finally, longitudinal shifts were also determined by random forest, with accuracy of the classification model notably lower (AR 70%; 80%) than storage temperature and location accuracy. Regarding OK samples, most biomarkers were gut microbiota including Otu27-*Bacteroides* and Otu38-*Prevotella* that decreased temporally. Regarding AR samples, BRD associated pathogens such as OTU35-*Pasteurellaceae* and Otu567-*Mycoplasmataceae* were observed to increase temporally.

Use of RT as A Sustainable Model for UL Control Samples

Procrustes analyses were used to estimate the consistency of the RT storage techniques effects compared to UL temperature storage on the beta diversity of samples based on Jaccard distance. Cross-sectional analysis (UL1AR-RT1AR, UL1OK-RT1OK, UL2AR-RT2AR, UL2OK-RT2OK) of storage technique demonstrated consistent effects on beta diversity **(Figure 5)**. All cross-sectional analyses of storage temperature effects demonstrated consistent movement at each

sampling location for both sampling timepoints. Longitudinal analysis (UL1AR-UL2AR, UL1OK-UL2OK, RT1AR-RT2AR, RT1OK-RT2OK) of the temporal effects on the nasal microbiome showed consistent movement for UL samples **(Figure 6 A)** but not RT samples **(Figure 6. B)**.

Discussion

Storage temperature and location both significantly impacted microbial alpha and beta diversity. Storage at RT for 7 days significantly decreased microbial diversity and observed species, and distinctly clustered on PCoA plots from UL storage samples based on both Jaccard and Bray-Curtis distance. Sampling location also significantly impacted both alpha and beta diversity of samples, observed by significant differences in microbial diversity, observed species and distinct clustering among PCoA plots base on Jaccard and Bray-Curtis distances. The longitudinal impact was not found significant for UL samples considering alpha diversity but was found statistically significantly different for RT samples. However, beta diversity measures for both Jaccard and Bray-Curtis distance found significant differences in the longitudinal effects with ANOSIM tests, although there was not distinct clustering found on the PCoA plots. Community structure at the phylum level was largely consistent among treatments, however, was found dissimilar at the genus and OTU levels as a reduction in the load of airway microbes and an increase in the load of gut microbes was observed. This is most likely due to the production of organic acids, such as lactic acid, from gut microbes, lowering the pH of the Amies buffer to favor genera such as *Enteroccocus* as well as the movement toward a more anaerobic environment favoring genera such as *Bacteroides,* also known to degrade mucins present in mucous, as observed at the genus and OTU levels for RT samples (Ramsey, Hartke, & Huycke, 2014). Furthermore, Random forest was found as a successful model to rank the importance of several respiratory and gut microbes found as

markers across storage temperature treatment, location and longitudinal effects. Primarily, airway and gut microbes were found as markers for UL temperature storage, however gut microbes were found to dominate the marker bacteria for RT samples. Sampling location and timepoint influenced the importance of bacteria within samples, shown by distinct markers for each location and timepoint when comparing UL samples. RT samples found fewer distinct markers across location and timepoint, revealing conditions in the self-enrichment culture technique were less defined than UL temperature samples. Procrustes analyses revealed consistent trends between UL and RT storage treatment for both locations and timepoints, suggesting the potential for RT storage as a self-enrichment technique with characterizable bias in future studies.

Conclusions

Overall community diversity, relative abundance, richness, and evenness were all shown to significantly decrease at 7 days of RT storage when compared to immediate UL temperature storage and were also found to be significantly different for location effects. The temporal effects on the nasal microbiome for alpha diversity were not found statistically significantly different, however ANOSIM revealed statistically significant differences in the beta diversity for UL and RT storage samples. Random forest was found as a successful model to rank the marker bacteria across treatments and biomarkers related to *Enterococcus* and *Pseudomonas* were consistently increased by RT storage technology. Furthermore, RT storage of samples for 7 days was found by the Procrustes analysis as a consistent model to culture bovine nasal swab samples. Overall, the novel self-enrichment room temperature storage technique was found to enrich specific microbiota but ultimately shifted the microbial structure of the “normal” respiratory community. Thus, future improvement and investigation into the novel self-enrichment technique that has the potential to enhance the concentration of some certain species relevant to the onset and diagnosis

of BRD for further downstream omics analysis (e.g. metatranscriptomics, metabolomic, etc.) is necessary to expand its uses.

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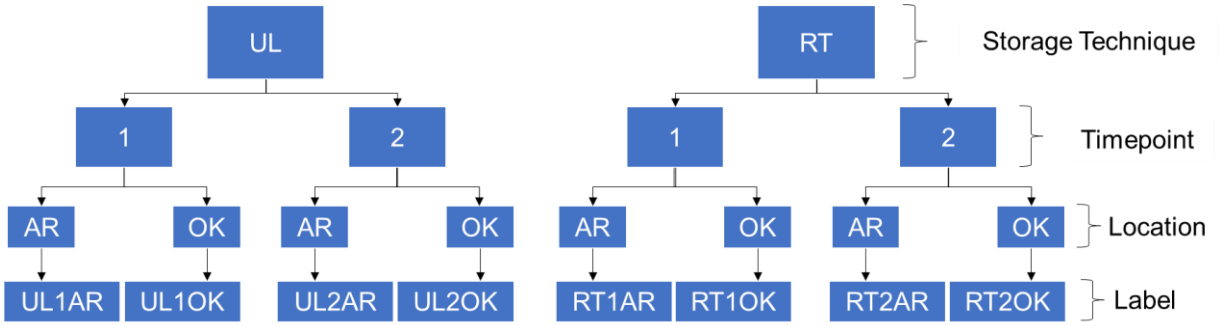
Appendix

Figure 1

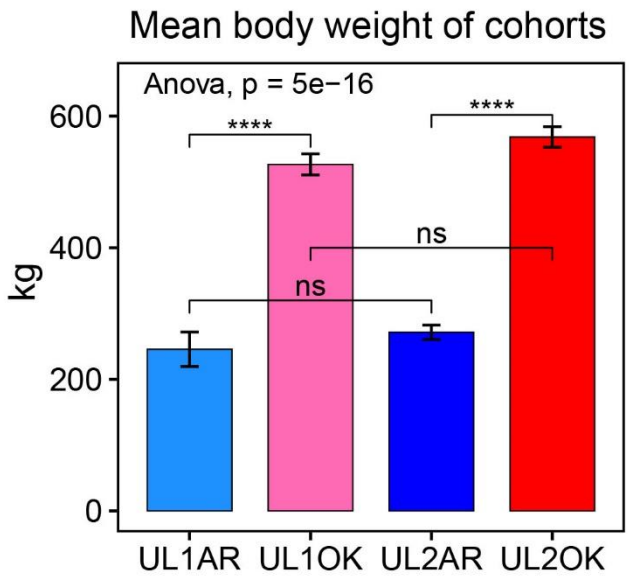
Experimental Design and Treatment Characterization



Note. Treatment characterization defining the labelling schematic for subjects' samples following their respective storage technique, sampling timepoint, location of sample collection, and defined label for analysis. Each treatment (label) was given 10 subjects that remained the same from timepoint 1 to timepoint 2.

Figure 2

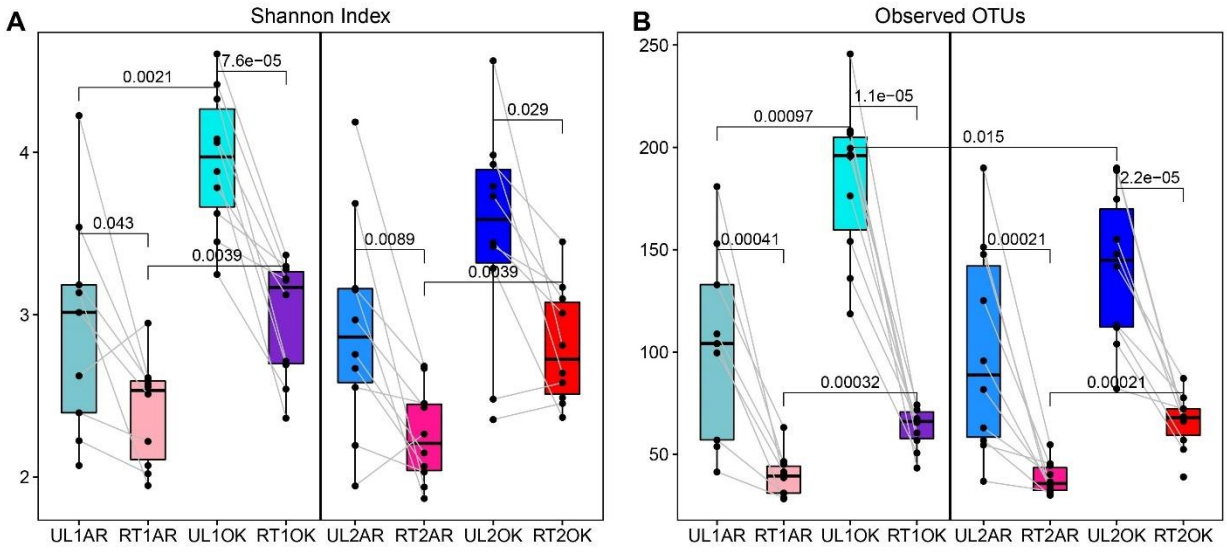
Mean Body Weight of Cohorts



Note. Mean body weight of calves at each location and sampling timepoint. Tukey Post Hoc test was used to test the statistical significance between all cohorts.

Figure 3

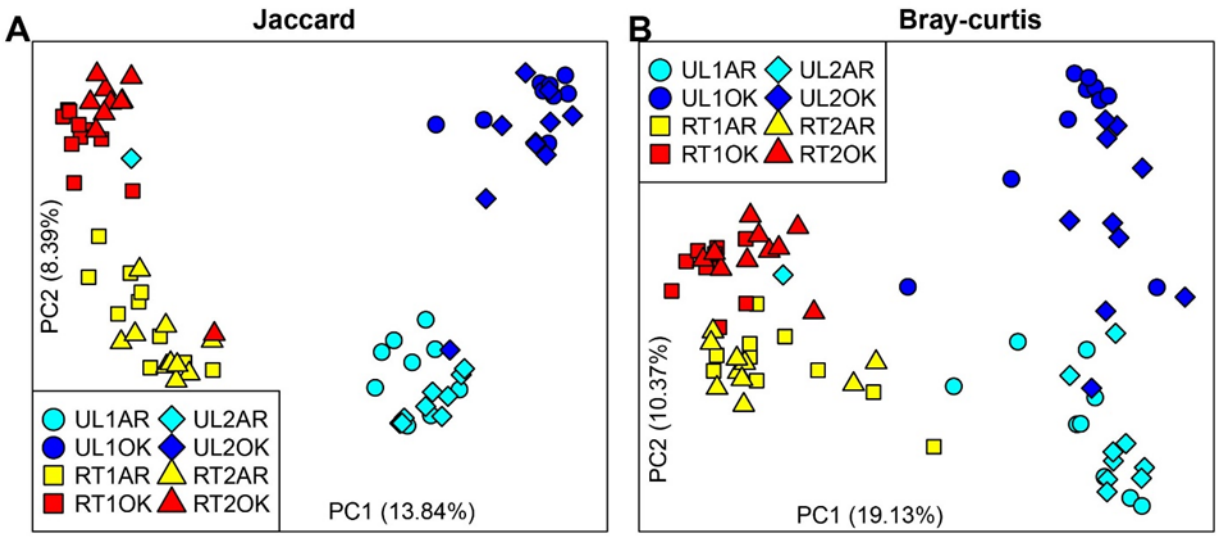
Pair-wise Comparison of Alpha Diversity for Storage Temperature, Location and Timepoint on the Nasal Microbiome



Note. Alpha diversity of nasal microbiome by storage temperature, location and sampling timepoint. Each point represents one sample. Samples connected by lines are from the same subject. Significant p-values from the Wilcoxon ranked sum test are labelled over bars.

Figure 4

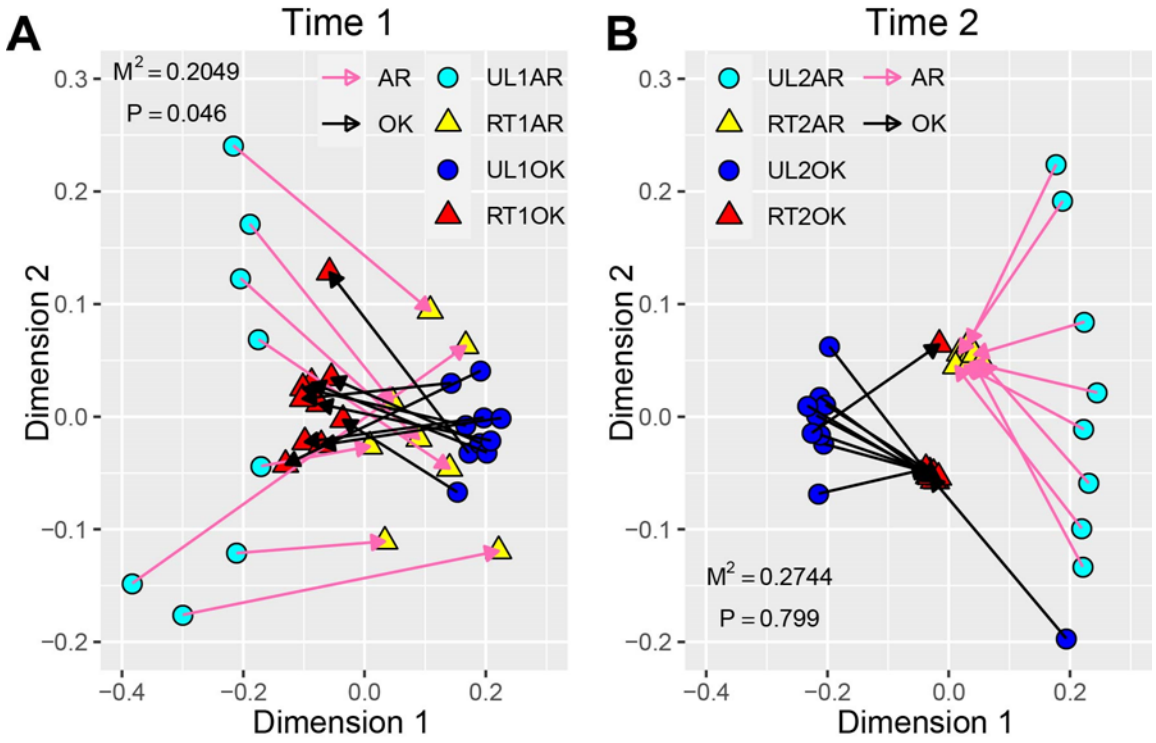
Beta Diversity of Storage Temperature, Location and Timepoint on the Nasal Microbiome



Note. Beta diversity of the nasal microbiome based on Jaccard and Bray-Curtis distances. Distinct clustering is observed for both distances across storage temperature and location effects. Each point represents a single sample.

Figure 5

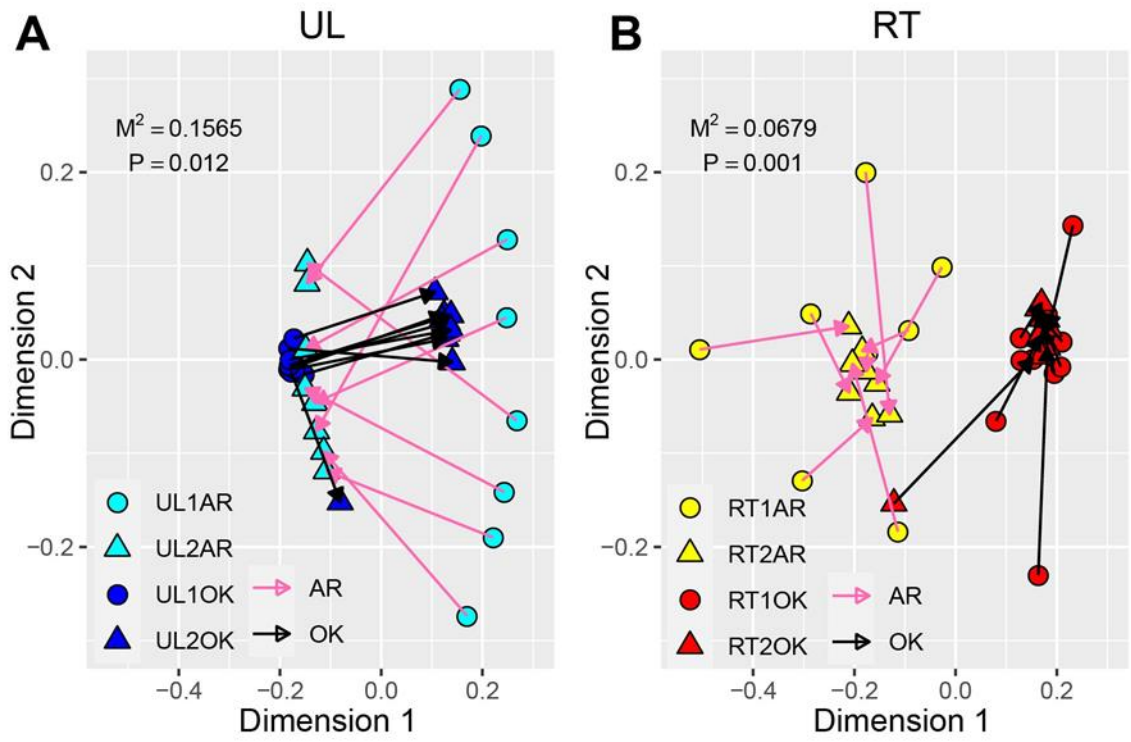
Procrustes Analysis of Storage Temperature Effects on the Nasal Microbiome



Note. Procrustes cross-sectional analysis of storage temperature effects on the nasal microbiome based on Jaccard distance. Consistent effects are observed by the synonymous directionality of subjects' samples connected by arrows. Samples corresponding to the same subject subjected to different treatments are connected by arrows.

Figure 6

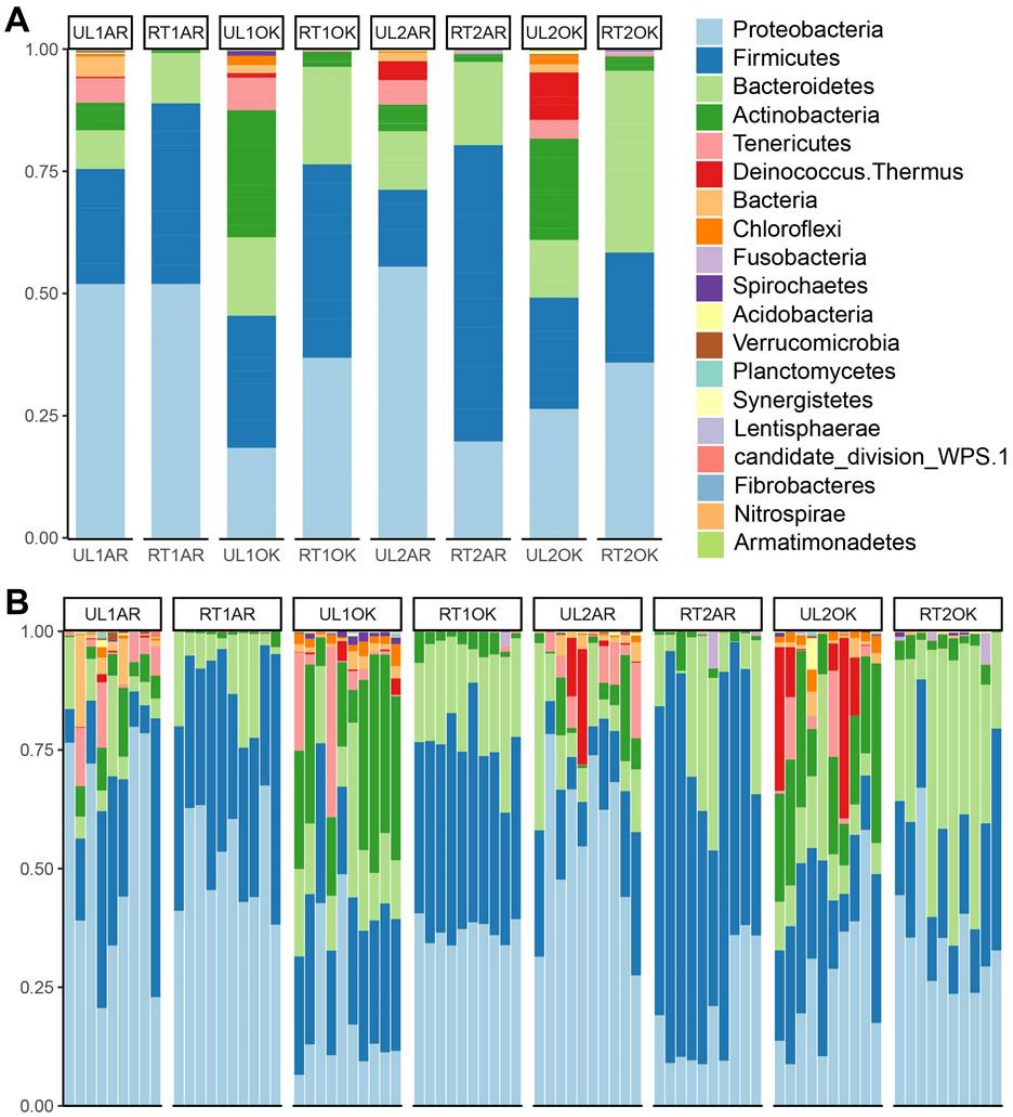
Procrustes Analysis of the Temporal Effects on the Nasal Microbiome



Note. Procrustes longitudinal analysis of sampling timepoint effects on the nasal microbiome. **(A).** Consistent trends in the temporal variation can be observed by the synonymous directionality of subjects' samples connected by arrows. **(B).** Temporal variation in RT samples was not found consistent.

Figure 7

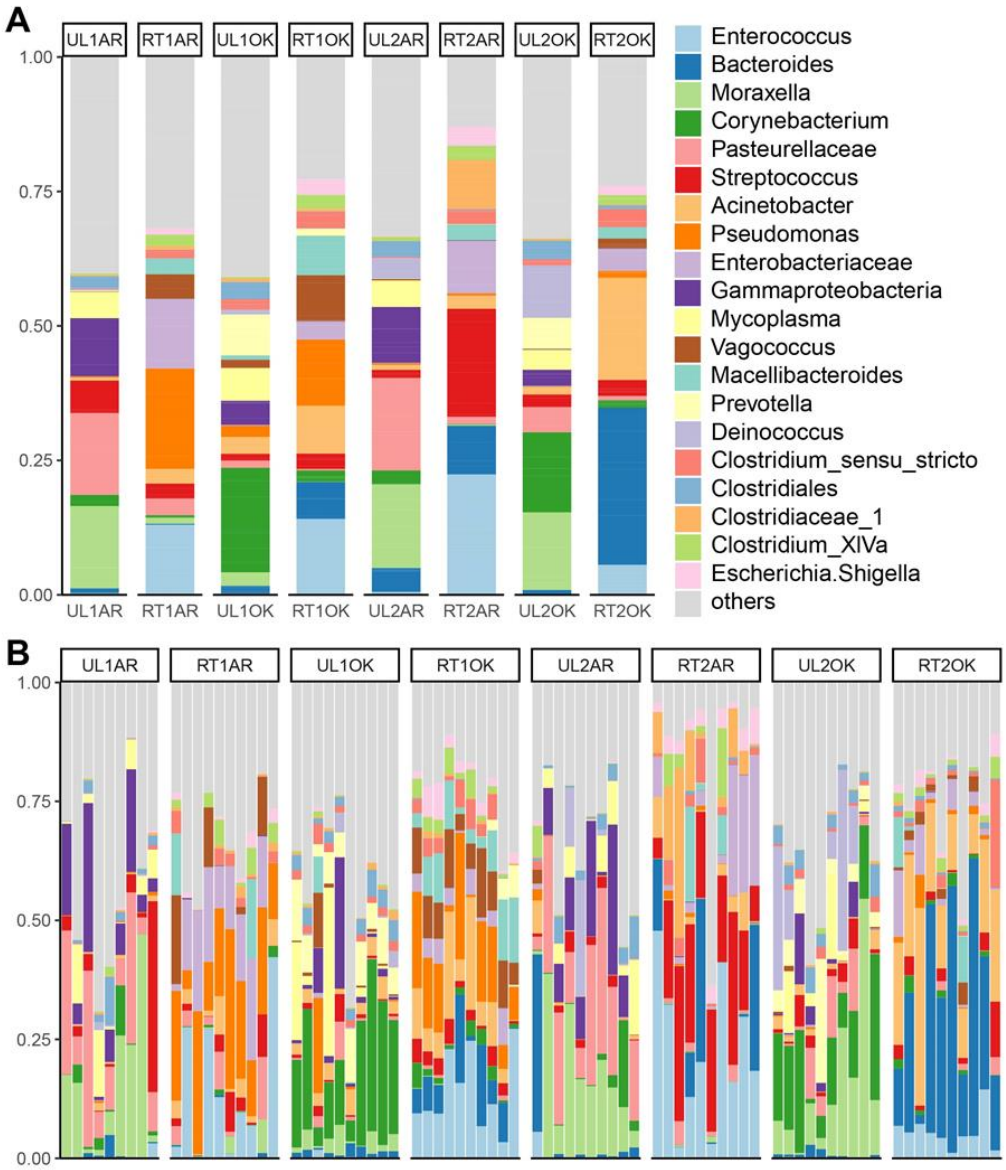
Community Structure of Storage Temperature, Location and Timepoint at the Phylum Level



Note. Nasal microbial composition as a function of relative abundance at the phylum level. **(A).** Average of the top 20 phyla. **(B).** Each column corresponds to a unique sample. Headings represent the treatment samples received, with 10 subjects in each treatment.

Figure 8

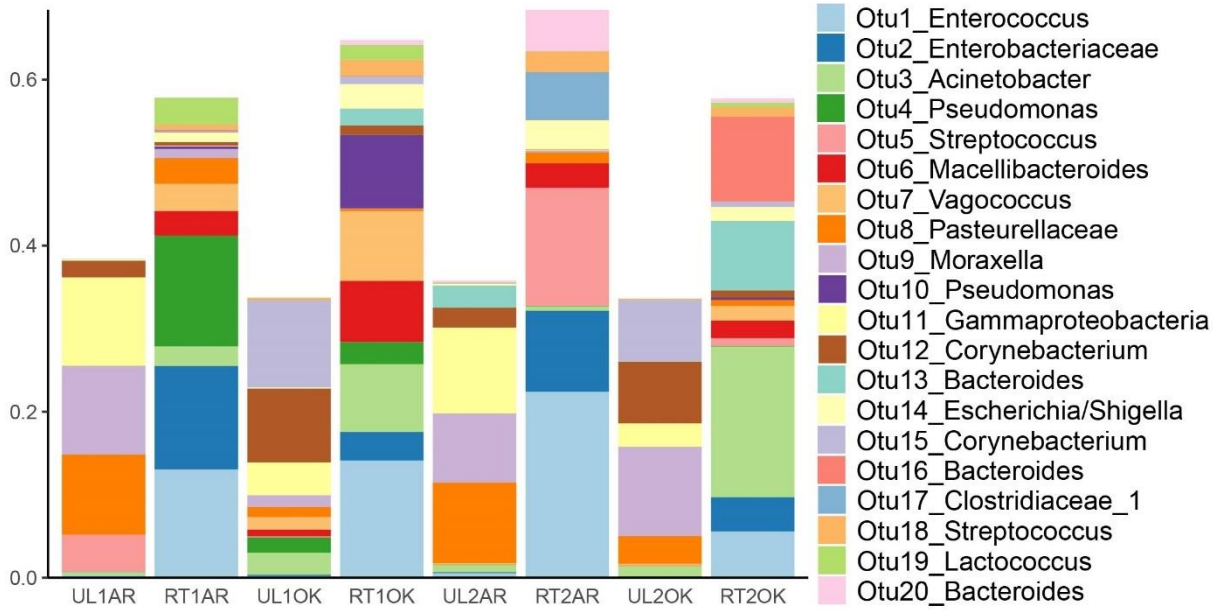
Community Structure of Storage Temperature, Location and Timepoint at the Genus Level



Note. Nasal microbial composition as a function of relative abundance at the genus level. **(A).** Average of the top 20 genera. **(B).** Each column corresponds to a unique sample. Headings represent the treatment samples received, with 10 subjects in each treatment.

Figure 9

Community Structure of Storage Temperature, Location and Timepoint at the OTU Level



Note. Nasal microbial composition for the average of the top 20 OTUs relative abundance found among samples in each treatment.

Table 1

Random Forest Biomarkers for Storage Temperature, Location and Longitudinal Analysis

Comparison	OTU	MDA	Treatment
UL1AR-RT1AR			
	Otu53 *Mycoplasma*	18.83	UL1AR
	Otu35 *Pasteurellaceae*	18.30	UL1AR
	Otu11 *Gammaproteobacteria*	18.14	UL1AR
	Otu143 *Chitinophagaceae*	16.90	UL1AR
	Otu30 *Moraxella*	15.40	UL1AR
	Otu158 *Bacteroides*	12.77	UL1AR
	Otu65 *Turicibacter*	12.30	UL1AR
	Otu157 *Planococcaceae*	12.17	UL1AR
	Otu132 *Bacillales*	11.77	UL1AR
	Otu206 *Nesterenkonia*	11.59	UL1AR
	Otu62 *Mycoplasma*	10.20	UL1AR
	Otu80 *Staphylococcus*	10.09	UL1AR
	Otu245 *Bacteroidetes*	9.75	UL1AR
	Otu96 *Phascolarctobacterium*	8.87	UL1AR
	Otu367 *Lactobacillus*	8.57	UL1AR
	Otu2 *Enterobacteriaceae*	17.79	RT1AR
	Otu4 *Pseudomonas*	14.60	RT1AR
	Otu76 *Clostridiales*	13.39	RT1AR
	Otu1 *Enterococcus*	12.89	RT1AR
	Otu36 *Lactococcus*	12.38	RT1AR
	Otu18 *Streptococcus*	11.95	RT1AR
	Otu74 *Aerococcus*	9.83	RT1AR
	Otu94 *Paenibacillus*	9.34	RT1AR
	Otu14 *Escherichia.Shigella*	9.19	RT1AR
	Otu357 *Pseudomonadaceae*	8.74	RT1AR
UL1OK-RT1OK			
	Otu191 *Petrimonas*	12.86	UL1OK
	Otu125 *Micrococcaceae*	12.85	UL1OK
	Otu116 *Clostridiaceae*	12.75	UL1OK
	Otu166 *Prevotella*	12.33	UL1OK
	Otu247 *Prevotellaceae*	12.10	UL1OK
	Otu173 *Gammaproteobacteria*	11.94	UL1OK
	Otu134 *Clostridium*	11.94	UL1OK
	Otu142 *Prevotella*	11.93	UL1OK

	Otu73 *Ornithinimicrobium*	11.90	UL1OK
	Otu156 *Succinivibrio*	11.89	UL1OK
	Otu187 *Prevotella*	11.88	UL1OK
	Otu65 *Turicibacter*	11.78	UL1OK
	Otu120 *Clostridiales*	11.68	UL1OK
	Otu38 *Prevotella*	11.58	UL1OK
	Otu96 *Phascolarctobacterium*	11.55	UL1OK
	Otu11 *Gammaproteobacteria*	9.97	UL1OK
	Otu196 *Facklamia*	9.45	UL1OK
	Otu199 *Alloprevotella*	9.13	UL1OK
	Otu1 *Enterococcus*	10.60	RT1OK
	Otu19 *Lactococcus*	10.60	RT1OK
	Otu14 *Escherichia.Shigella*	9.90	RT1OK
	Otu72 *Paraeggerthella*	9.57	RT1OK
	Otu85 *Brevundimonas*	9.22	RT1OK
	Otu51 *Facklamia*	9.12	RT1OK
	Otu7 *Vagococcus*	9.04	RT1OK
UL2AR-RT2AR			
	Otu9 *Moraxella*	15.37	UL2AR
	Otu209 *Bacteroides*	14.37	UL2AR
	Otu76 *Clostridiales*	14.29	UL2AR
	Otu81 *Carnobacteriaceae*	14.19	UL2AR
	Otu53 *Mycoplasma*	14.00	UL2AR
	Otu242 *Alistipes*	13.33	UL2AR
	Otu35 *Pasteurellaceae*	11.95	UL2AR
	Otu11 *Gammaproteobacteria*	11.79	UL2AR
	Otu96 *Phascolarctobacterium*	11.77	UL2AR
	Otu160 *Ruminococcaceae*	11.68	UL2AR
	Otu102 *Romboutsia*	11.18	UL2AR
	Otu30 *Moraxella*	11.09	UL2AR
	Otu261 *Bacteroidetes*	10.52	UL2AR
	Otu42 *Enhydrobacter*	10.39	UL2AR
	Otu204 *Mogibacterium*	9.43	UL2AR
	Otu5 *Streptococcus*	18.51	RT2AR
	Otu12 *Corynebacterium*	17.77	RT2AR
	Otu14 *Escherichia.Shigella*	17.40	RT2AR
	Otu18 *Streptococcus*	15.81	RT2AR
	Otu1 *Enterococcus*	15.44	RT2AR
	Otu2 *Enterobacteriaceae*	15.07	RT2AR
	Otu195 *Lactococcus*	12.96	RT2AR
	Otu68 *Dermabacteraceae*	11.90	RT2AR

	Otu86 *Clostridium sensu stricto*	11.11	RT2AR
	Otu17 *Clostridiaceae*	9.33	RT2AR
UL2OK-RT2OK			
	Otu114 *Lachnospiraceae*	14.89	UL2OK
	Otu65 *Turicibacter*	14.81	UL2OK
	Otu95 *Dietzia*	14.69	UL2OK
	Otu73 *Ornithinimicrobium*	14.59	UL2OK
	Otu125 *Micrococcaceae*	14.49	UL2OK
	Otu9 *Moraxella*	13.26	UL2OK
	Otu204 *Mogibacterium*	12.14	UL2OK
	Otu106 *Sphaerobacteraceae*	12.06	UL2OK
	Otu211 *Dietzia*	11.60	UL2OK
	Otu135 *Roseburia*	11.56	UL2OK
	Otu102 *Romboutsia*	11.20	UL2OK
	Otu136 *Lachnospiraceae*	10.85	UL2OK
	Otu11 *Gammaproteobacteria*	10.67	UL2OK
	Otu166 *Prevotella*	10.66	UL2OK
	Otu218 *Clostridiales*	10.51	UL2OK
	Otu168 *Clostridiales*	10.35	UL2OK
	Otu15 *Corynebacterium*	10.28	UL2OK
	Otu3 *Acinetobacter*	15.08	RT2OK
	Otu21 *Clostridium*	13.95	RT2OK
	Otu1 *Enterococcus*	13.72	RT2OK
	Otu14 *Escherichia.Shigella*	12.93	RT2OK
	Otu85 *Brevundimonas*	11.54	RT2OK
	Otu2 *Enterobacteriaceae*	11.27	RT2OK
	Otu108 *Peptostreptococcus*	11.24	RT2OK
	Otu72 *Paraeggerthella*	10.54	RT2OK
UL1AR-UL1OK			
	Otu15 *Corynebacterium*	13.40	UL1OK
	Otu166 *Prevotella*	13.34	UL1OK
	Otu191 *Petrimonas*	13.32	UL1OK
	Otu187 *Prevotella*	13.25	UL1OK
	Otu247 *Prevotellaceae*	13.25	UL1OK
	Otu173 *Gammaproteobacteria*	12.74	UL1OK
	Otu38 *Prevotella*	12.74	UL1OK
	Otu142 *Prevotella*	12.63	UL1OK
	Otu65 *Turicibacter*	12.26	UL1OK
	Otu120 *Clostridiales*	11.87	UL1OK
	Otu3 *Acinetobacter*	11.37	UL1OK
	Otu210 *Prevotella*	10.78	UL1OK

	Otu233 *Firmicutes*	10.74	UL1OK
	Otu169 *Prevotella*	10.50	UL1OK
	Otu224 *Firmicutes*	10.26	UL1OK
	Otu199 *Alloprevotella*	10.22	UL1OK
	Otu73 *Ornithinimicrobium*	10.10	UL1OK
	Otu236 *Ruminococcaceae*	10.08	UL1OK
	Otu188 *Alloprevotella*	10.07	UL1OK
	Otu41 *Clostridium sensu stricto*	10.05	UL1OK
	Otu104 *Bacteroides*	9.91	UL1OK
	Otu53 *Mycoplasma*	13.78	UL1AR
	Otu35 *Pasteurellaceae*	13.46	UL1AR
	Otu143 *Chitinophagaceae*	11.43	UL1AR
	Otu80 *Staphylococcus*	10.37	UL1AR
UL2AR-UL2OK			
	Otu106 *Sphaerobacteraceae*	16.81	UL2OK
	Otu95 *Dietzia*	15.26	UL2OK
	Otu218 *Clostridiales Incertae Sedis XI*	14.31	UL2OK
	Otu15 *Corynebacterium*	14.19	UL2OK
	Otu211 *Dietzia*	13.94	UL2OK
	Otu41 *Clostridium sensu stricto*	13.69	UL2OK
	Otu166 *Prevotella*	13.28	UL2OK
	Otu120 *Clostridiales*	13.25	UL2OK
	Otu168 *Clostridiales*	13.09	UL2OK
	Otu138 *Actinomycetaceae*	13.06	UL2OK
	Otu73 *Ornithinimicrobium*	13.02	UL2OK
	Otu38 *Prevotella*	12.64	UL2OK
	Otu136 *Lachnospiraceae*	11.09	UL2OK
	Otu187 *Prevotella*	10.83	UL2OK
	Otu208 *Clostridiales*	10.78	UL2OK
	Otu237 *Clostridiales Incertae Sedis XI*	10.77	UL2OK
	Otu169 *Prevotella*	10.67	UL2OK
	Otu199 *Alloprevotella*	10.63	UL2OK
	Otu193 *Firmicutes*	10.51	UL2OK
	Otu148 *Clostridiales Incertae Sedis XI*	10.38	UL2OK
	Otu142 *Prevotella*	10.35	UL2OK
	Otu223 *Prevotella*	10.28	UL2OK
	Otu230 *Bifidobacterium*	10.25	UL2OK
	Otu12 *Corynebacterium*	9.86	UL2OK
	Otu42 *Enhydrobacter*	9.87	UL2AR
UL1AR-UL2AR			
	Otu242 *Alistipes*	7.87	UL2AR

	Otu699 *Actinomycetales*	7.31	UL2AR
	Otu55 *Flavobacteriaceae*	7.08	UL2AR
	Otu653 *Saccharofermentans*	7.00	UL2AR
	Otu567 *Mycoplasmataceae*	6.63	UL2AR
	Otu411 *Bacteroides*	5.81	UL2AR
	Otu531 *Clostridiales*	5.56	UL2AR
	Otu24 *Bacteroides*	4.79	UL2AR
	Otu152 *Arthrobacter*	12.94	UL1AR
	Otu367 *Lactobacillus*	11.80	UL1AR
	Otu32 *Pseudomonas*	10.80	UL1AR
	Otu116 *Clostridiaceae*	10.38	UL1AR
	Otu515 *Bacillaceae*	8.70	UL1AR
	Otu80 *Staphylococcus*	8.66	UL1AR
	Otu157 *Planococcaceae*	7.94	UL1AR
	Otu206 *Nesterenkonia*	7.86	UL1AR
	Otu213 *Bacillus*	7.68	UL1AR
	Otu778 *Sphingomonas*	7.03	UL1AR
	Otu527 *Ruminococcaceae*	6.93	UL1AR
	Otu40 *Comamonas*	6.38	UL1AR
	Otu181 *Porphyromonadaceae*	5.98	UL1AR
	Otu311 *Ruminobacter*	5.89	UL1AR
	Otu797 *Lachnospiraceae*	5.87	UL1AR
	Otu269 *Lachnospiraceae*	5.46	UL1AR
	Otu54 *Stenotrophomonas*	5.02	UL1AR
UL1OK-UL2OK			
	Otu218 *Clostridiales Incertae Sedis XI*	13.18	UL2OK
	Otu301 *Clostridiales*	12.46	UL2OK
	Otu129 *Streptococcus*	7.27	UL2OK
	Otu256 *Prevotella*	7.07	UL2OK
	Otu27 *Bacteroides*	12.67	UL1OK
	Otu269 *Lachnospiraceae*	12.28	UL1OK
	Otu364 *Bacteroides*	11.65	UL1OK
	Otu184 *Veillonellaceae*	11.58	UL1OK
	Otu236 *Ruminococcaceae*	11.57	UL1OK
	Otu241 *Treponema*	11.09	UL1OK
	Otu104 *Bacteroides*	10.62	UL1OK
	Otu350 *Bacteria*	9.80	UL1OK
	Otu413 *Bacteroidales*	9.74	UL1OK
	Otu439 *Lachnospiraceae*	9.66	UL1OK
	Otu235 *Bacteria*	9.47	UL1OK
	Otu210 *Prevotella*	9.30	UL1OK

Otu278 *Clostridium IV*	9.01	UL1OK
Otu484 *Alloprevotella*	8.78	UL1OK
Otu3 *Acinetobacter*	7.97	UL1OK
Otu409 *Lachnospiraceae*	7.61	UL1OK
Otu583 *Lachnospiraceae*	7.25	UL1OK
Otu191 *Petrimonas*	6.89	UL1OK
Otu38 *Prevotella*	6.36	UL1OK
Otu200 *Bacteroidales*	6.27	UL1OK
Otu220 *Olsenella*	6.16	UL1OK

Note. Random forest results for compared treatments across storage temperature, location and time. Comparisons of biomarkers are ranked from the top with the highest correlating mean decreased accuracy value (MDA) to the bottom for the lowest MDA value. Treatments are grouped within each comparison.