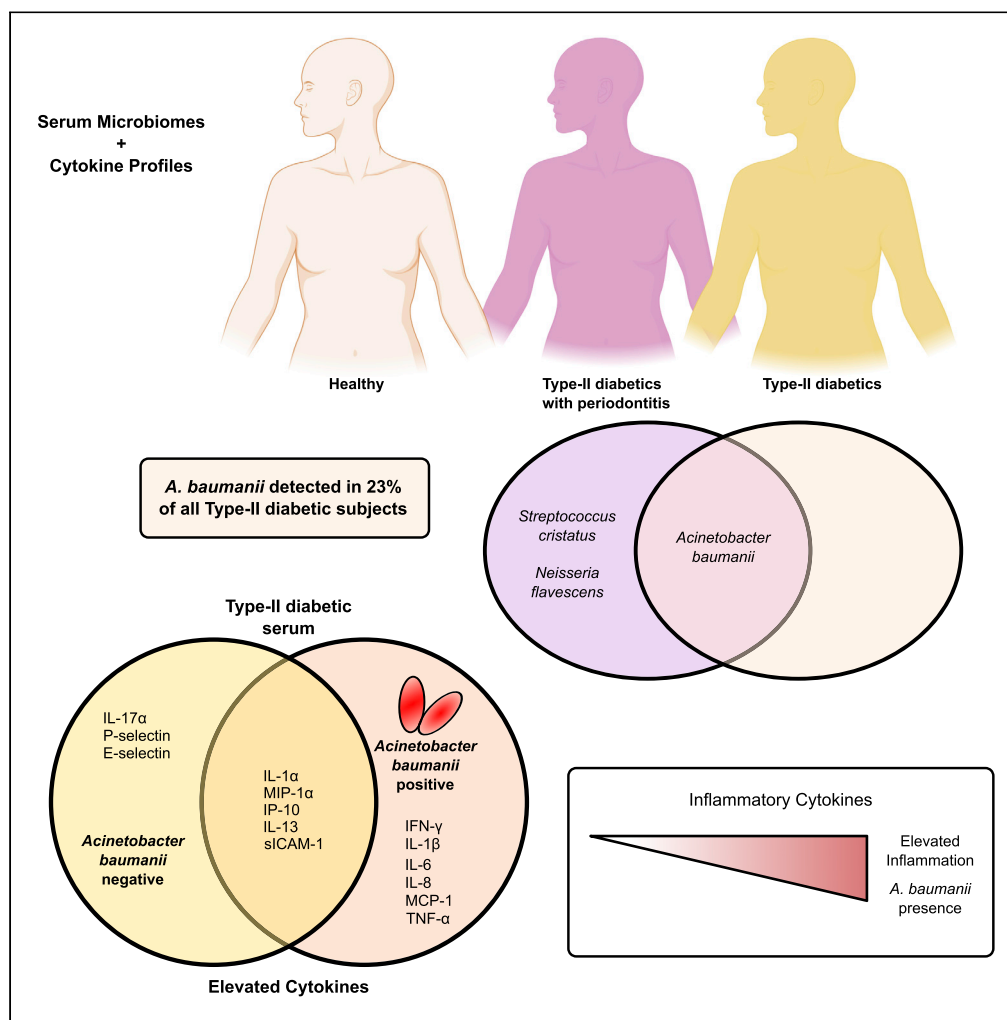# iScience

Article

Impaired host response and the presence of *Acinetobacter baumannii* in the serum microbiome of type-II diabetic patients

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Highlights

Acinetobacter baumanii was found in the serum of 23% type-II diabetic patients

Minor but significant serum microbiome changes between healthy vs type-II diabetic patients

Proinflammatory cytokines were elevated in *A. baumanii*-positive serum samples

A. baumanii + serum cytokine profiles were consistent with chronic inflammation

Article

Impaired host response and the presence of *Acinetobacter baumannii* in the serum microbiome of type-II diabetic patients

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Summary

Type II diabetes (T2D) affects over 10% of the US population and is a growing disease worldwide that manifests with numerous comorbidities and defects in inflammation. This dysbiotic host response allows for infection of the host by numerous microorganisms. In the course of T2D disease, individuals can develop chronic infections including foot ulcers and periodontitis, which lead to further complications and opportunistic infections in multiple body sites. In this study, we investigated the serum of healthy subjects and patients with T2D with (T2DP) or without periodontitis for both microbiome signatures in addition to cytokine profiles. Surprisingly, we detected the presence of *Acinetobacter baumanii* in the serum of 23% individuals with T2D/T2DP tested. In T2DP, IL-1β, TNF-α, MCP-1, IL-6, IL-8, and IFN-γ were significantly elevated in ABC-positive subjects. As an emerging pathogen, *A. baumanii* infection represents a risk for impaired inflammation and the development of comorbidities in subjects with T2D.

Introduction

Type-II diabetes (T2D) is a chronic inflammatory disease affecting 10.5% of the US population, with rising prevalence both in the United States and worldwide (Whiting et al., 2011; National Diabetes Statistics Report | Data & Statistics | Diabetes | CDC, 2020). Individuals with T2D typically have numerous comorbidities and are at a greater risk of both opportunistic and nosocomial infections, which can result in significant morbidity, including amputations, and lost quality of life (QOL) measures (Vardakas et al., 2007; Erben et al., 2013; McDonald et al., 2014; Ferlita et al., 2019; Kim et al., 2019). Thus, care must be taken to prevent and monitor the infection status of these individuals, as the development of diabetic mucosal and skin lesions across the body can impact the host immune and diabetic responses. Key infection prevention includes minimizing portals of entry and colonization by numerous pathogens. One example of such a portal of entry is the emergence of diabetic foot ulcers, which can develop diabetic foot infections (DFIs). Roughly 15% of adults with T2D develop foot ulcers and ~14% of these subjects undergo amputation, with the remainder needing extensive debridement, negative pressure treatments, and other therapies including antibiotics, which result in prolonged hospitalization time, lengthy healing duration, and increased treatment costs (Reiber et al., 1998; Driver et al., 2010).

In addition to DFIs, subjects with T2D are ~3 times more likely to develop periodontitis (Emrich et al., 1991; Wu et al., 2020). Periodontitis is an inflammatory oral disease accompanied by a polymicrobial biofilm infection that can lead to tissue damage, chronic lesions, and tooth/bone loss in the oral cavity (Irfan et al., 2001; Genco et al., 2020; Mann et al., 2020). During periodontal infection, the healthy oral microbiome, especially in the subgingival space (along teeth below the gumline, including periodontal ligament, epithelium, alveolar bone, and connective tissues), transitions to one enriched with several opportunistic pathogens that exacerbate inflammation and host tissue damage both locally and systemically (Socransky et al., 1998; Griffen et al., 2012; Hajishengallis and Lamont, 2012; Roberts and Darveau, 2015; Nowicki et al., 2018; Curtis et al., 2020). Although protective, acute inflammation is a helpful immune response, it is characterized by rapid activation of inflammation and subsequent return to homeostasis (resolution). Unresolved inflammation that fails to control the trigger leads to chronic lesions and is a hallmark of both periodontitis and T2D.

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More acute bacteremia can manifest in diseases such as endocarditis or other distal infections by oral microbes (Lockhart and Durack, 1999; Parahitiyawa et al., 2009; Carrizales-Sepúlveda et al., 2018). Various systemic diseases are in fact influenced by microbial metabolism and host interactions. "How" and "why" immune system imbalance fails to control microbial pathogenic transition remains an active area of investigation. Increasing evidence implicates periodontal diseases, especially periodontitis in adults, as a potential risk factor for increased morbidity and mortality from systemic conditions including diabetes, cardiovascular diseases, adverse pregnancy outcomes, and others (Genco et al., 2020; Kleinstein et al., 2020b). This is due to two plausible mechanisms: a direct pathway leading to migration of the bacteria to distal organs and an indirect pathway leading to production of microbial metabolites and/or activating inflammatory mediators that activate the immune system locally and systemically. This is likely made possible as periodontitis presents as a chronic inflammatory condition of the tooth-supporting structures occurring with oral microbiome dysbiosis and continuous inflammatory burden. Notably, oral microbes have also been detected (Dowd et al., 2008; Gardner et al., 2013) and isolated from DFIs (Bowler and Davies, 1999; Citron et al., 2007). Thus, we hypothesized that patients with T2D might be at a greater risk of extraoral infection and may exhibit changes in microbiota that transiently occupy their bloodstream. We further hypothesized that these changes would be even more apparent in patients with T2D with periodontitis (T2DP) and that these patients may carry potential DFI-colonizing oral species in their bloodstream. Our initial investigations toward these hypotheses utilized samples derived from previous work on resolution of inflammation in T2D (Freire et al., 2017) and are reported here, with unexpected results.

To investigate the possibility of oral species presence in the bloodstream, we compared the serum microbiomes of subjects with T2D and T2DP versus a healthy cohort. Unexpectedly, we detected *Acinetobacter baumanii* in 23% of the subjects with T2D/P in our study. *A. baumanii* is an emerging infectious pathogen (Villar et al., 2014) notorious for its evolving antibiotic resistances (Gootz and Marra, 2008; Vázquez-López et al., 2020) and nosocomial (Dijkshoorn et al., 2007; Ayoub Moubareck and Hammoudi Halat, 2020) and bloodstream infections (Peleg et al., 2008) and is part of a cluster of *Acinetobacter* species with similar clinical manifestations known as the AB or ABC complex (Gerner-Smidt and Tjernberg, 1993), which are highly similar via ribotyping. ABC complex organisms present several particular risks to subjects with T2D, specifically higher mortality rates for those with bacteremia (Leão et al., 2016) and for those with higher blood glucose concentrations (Leung and Liu, 2019). Patients infected with *A. baumanii* after burns were 9.8 times more likely to develop glucose intolerance, and ~3 times more patients with T2D with burns developed *A. baumanii* infections (27%) than patients without T2D (8.5%) (Furniss et al., 2005). Additionally, *A. baumanii* has also been observed in DFIs, particularly alongside other multidrug-resistant pathogens, presenting a grave concern for individuals with T2D (Castellanos et al., 2019; Henig et al., 2020). In addition to DFIs, ABC complex organisms can also spread through the body and form infections in numerous organs (Peleg et al., 2008; Al-Anazi and Al-Jasser, 2014). *A. baumanii* in a mouse pneumonia model led to increased proinflammatory cytokines, reduced neutrophil infiltration into the lung, and increased extrapulmonary dissemination (Qiu et al., 2009). In T2D, neutrophils present failure in early chemotaxis, but a hyper-inflammatory feedback loop emerges to compensate the initial myeloid cell failure (Kleinstein et al., 2020a).

In this work, we first investigated whether or not we could detect signatures of oral bacteria; our findings indicated minor, yet significant, microbiome compositional changes between healthy cohort and patients with T2D and T2DP. We discovered that nearly one-fourth of the subjects with T2DP in our study contained sequence reads identical to that of *A. baumanii.* The specific presence of *A. baumanii* was confirmed by further clone library and 16S rDNA Sanger sequencing. In addition, we assayed the host response by cytokine profiles of sera. When stratifying *A. baumanii*-positive T2D versus *A. baumanii*-negative T2D samples we observed unique cytokine profiles, suggesting a specific microbial impact on systemic inflammation. Although our methodology cannot indicate if *A. baumanii* was transiently present or a subclinical colonizer of our T2DP cohort, we propose that these data reveal a much greater risk of currently "uninfected" individuals with T2D for *A. baumanii* exposure and infection than previously understood and should justify more intensive and thorough investigation into AB complex colonization of patients with T2D.

Results

Patient demographics

Serum samples were generated from the population described in Table 1. A total of 81 subjects were investigated in this study, 57 with T2D, of which 29 had periodontitis (T2DP) as defined by the American

Table 1. Study subject demographics

	Healthy (N = 24)	Type II diabetes (N = 57)	Total (N = 81)	p value
Age (mean in years ± SD)	46 ± 10.72	55 ± 10.78	52.78 ± 11.40	<0.001
Gender (no., %)				
Male	13 (54%)	29 (51%)	42 (51.85%)	
Female	11 (46%)	28 (49%)	39 (48.15)	0.45
Ethnicity				
Caucasian	15 (63%)	25 (43.86%)	40 (49.38%)	
Hispanic	2 (8%)	7 (12.28%)	9 (11.11%)	
African American	6 (25%)	23 (40.35%)	29 (35.80%)	
Asian	0	2 (3.51%)	2 (2.47%)	
Other	1 (4%)	0	1 (1.23%)	0.16
Smoking status (no., %)				
Smokers	1 (4%)	5 (8.77%)	6 (7.41%)	
Former smokers	0	21 (36.84%)	21 (25.93%)	
Never smokers	23 (96%)	31 (54.39%)	54 (66.67%)	0.02
BMI (kg/m^2; mean ± SD)	29.34 ± 2.60	32.93 ± 6.02	31.87 ± 5.48	<0.01
Blood cholesterol (mg/dL; mean ± SD)	148.89 ± 36.71	211.73 ± 78.69	194.01 ± 74.84	<0.001
HbA1c (%; mean ± SD)	–	7.48 ± 1.61	–	–
Blood Glucose (mg/dL; mean ± SD)	99.25 ± 19.29	190.84 ± 60.58	163.70 ± 66.69	<0.0001
Periodontal condition (no., %)				
Healthy	24 (100%)	7 (12.28%)	31 (38.27%)	
Mild	0	15 (26.32%)	15 (18.52%)	
Moderate	0	11 (19.30%)	11 (13.58%)	
Severe	0	12 (21.05%)	12 (14.81%)	
Gingivitis	0	10 (17.54%)	10 (12.35%)	
Stable periodontitis	0	2 (3.51%)	2 (2.47%)	<0.0001
ABC positive (no., %)				
Positive	0	13 (22.81%)	13 (16.05%)	
Negative	19 (79%)	29 (50.88%)	48 (59.26%)	
Not tested	5 (21%)	15 (26.32%)	20 (24.69%)	0.02
Neutrophil count (million cells, mean ± SD)	74.82 ± 58.56	158.22 ± 90.08	131.90 ± 94.51	<0.0001
Monocyte count (million cells, mean ± SD)	63.79 ± 50.35	113.91 ± 60.09	99.22 ± 63.28	<0.01

p values calculated by unpaired t tests or χ2 (two-sided p values; italicized p < 0.05 significant).
BMI, body-mass index; ABC, *A. baumanii*; %, percentage; mg/dL, milligrams per deciliter; kg/m^2, kilograms per square meter.
Smoking classification according to CDC National Health Interview Survey (NHIS).

Association of Diabetes (American Diabetes Association, 2015) and American Association of Periodontology (AAP) (Armitage, 1999) criteria, respectively. All T2D donors were poorly controlled diabetic subjects who had not taken antimicrobials, non-steroidal anti-inflammatory drugs, or insulin sensitizers within three months. Diagnostic serum glucose measures were taken for all subjects, and HbA1C was measured for diabetic patients to stage disease status. As expected, poorly controlled diabetic subjects (HbA1c% >6.5%) showed significantly elevated glucose levels (p < 0.0001; Table 1). There were no significant differences in gender or ethnicity between healthy volunteers and diabetic patients. Diabetic patients showed elevated body mass index and tended to be older than healthy controls (p < 0.05), consistent with known disease biology. Periodontal condition was further stratified among the subjects according to the severity of irreversible tissue loss (mild, moderate, severe) and reversible lesions called gingivitis according to AAP (Armitage, 1999) criteria. Periodontal condition was significantly worse in diabetic compared with healthy

subjects (p < 0.0001). All healthy subjects had healthy periodontal condition compared with only 11% diabetic patients, with 15% diabetic subjects suffering from severe periodontitis. Current and former smokers were near uniformly part of the T2D and T2DP groups with only one current smoker in the healthy group. The entire study population had a median age range of 53 ± 11 years, and all subjects were outpatient and thus not part of an extended hospital stay cohort.

Microbiome analysis controls for low template sequencing

Data generated here was part of a *post hoc* study of serum samples collected for a previous study (Freire et al., 2017) and thus not available in the larger volumes ideal for 16S microbiome dataset generation. Low template concentrations can produce microbiome signatures not derived from that in the biological samples due to amplification of contaminating genomic DNA (gDNA) in sterile buffers and reagents (Kennedy et al., 2014; Salter et al., 2014; Kim et al., 2017), which often manifests as aquatic species signatures, many of which are not compatible with growth in the human host (i.e., many do not even grow at 37°C) (Kim et al., 2017). A description of no-template/reagent-only control samples is provided in the Methods. Data within this study are provided as either minimally filtered (mitochondrial, human, and chloroplast aligned reads removed) or strict filtered (reads removed based on homology with reagent-only controls) and indicated as such throughout the study. All sequence data are made available at NIH SRA Bioproject PRJNA664044, and all scripts used for data generation, filtering, and analyses are provided in the Supplemental Information.

We were able to generate abundant data from these samples with careful consideration paid to controls for amplification bias and background bacterial gDNA contamination of commercial sterile reagents. First, we utilized amplification of a mock standard library of 8 bacterial and 2 fungal species (Figures S1A and S1B) at 10 and 1 ng of total template, which indicated that our library preparation protocol was suitable for species-level analysis where possible by V1-V3 sequence. Second, we utilized 13 separate no-template/reagent-only controls, which were subjected to our sequence analysis pipeline, revealing frequently encountered contaminating taxa. These taxa were filtered from each human-derived sample in the strict-filtered datasets only.

V1-V3 16S microbiome diversity analyses reveal minor but significant differences between diabetic and nondiabetic groups

Serum and control samples were subject to bead beater lysis and gDNA purification. Purified gDNA was amplified using the V1-V3-targeted 27F-519R primer pair (Stackebrandt and Goodfellow, 1991; Turner et al., 1999). Quality trimmed reads were subjected to DADA2 analysis (Callahan et al., 2016) and aligned to the SILVA database (Quast et al., 2013) to determine species-level identity where possible. Rarefaction curve analysis was performed to ensure adequate sequencing depth (Figure S2), and resequencing of each Illumina index was performed to generate greater sequencing depth without further template amplification. Run-to-run variability between next-generation sequencing was found to be insignificant (Figure S3). Data analysis and comparisons were performed via QIIME2 (Bolyen et al., 2019) as detailed in the Methods.

We first quantified alpha and beta diversity between the healthy, T2D, and T2DP subject groups for our strict filtered (Figures 1 and S7) and minimally filtered data (Figure S4). For both datasets, species richness for T2D and T2DP groups were significantly different than the healthy cohort, which was also significantly different when compared with T2DP in each dataset for species evenness (T2D more rich, T2DP less rich, and less even). Beta diversity was significantly less for healthy subjects versus T2D and greater when compared with T2DP for either minimal or strict filtered datasets. Thus, we observed minor but significant shifts in microbiome composition between subject groups.

To begin investigating which taxa contributed to this composition difference we first looked at a phylum-level display of our taxonomically assigned data (Figure 2). Figures 2A and 2B, respectively, indicate phylum-level data for strict versus minimal filtered datasets. Similar trends in phyla distribution were evident for either dataset and reveal minor phylum-level changes between subject groups.

MED and DADA2 analysis reveal the presence of *Acinetobacter baumanii*

Given our initial investigation into whether or not there were differences between T2D and T2DP microbiota and a preliminary hypothesis that oral taxa would be enriched in T2DP serum, we first used a minimal entropy decomposition (MED) analysis identical to the one used previously to characterize oral
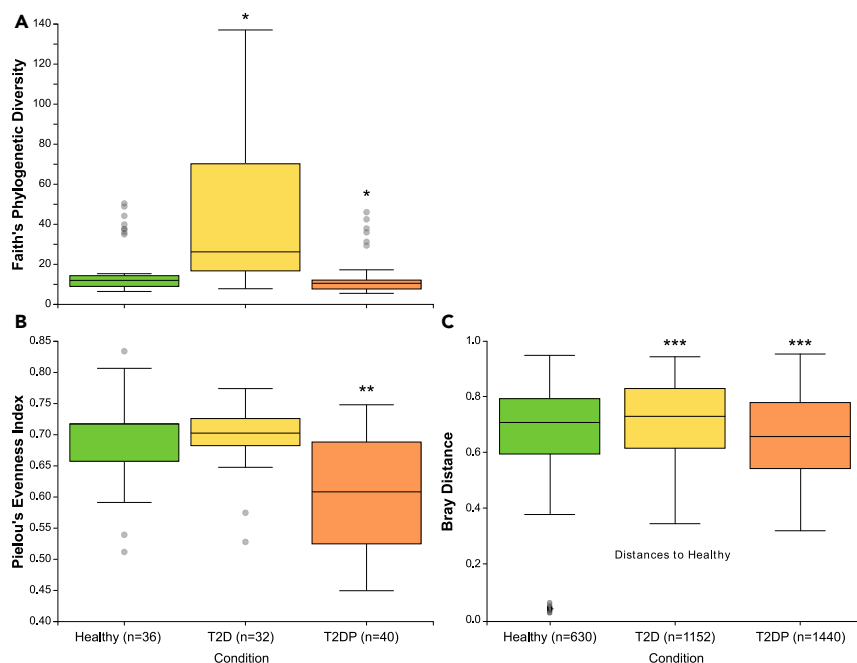
Figure 1. 16S alpha and beta diversity of serum microbiomes changes with subject status

(A–C) Species richness is displayed based on Faith's phylogenetic diversity (Faith, 1992) (A), and species evenness is based on Pielou's evenness index (Pielou, 1966) (B) for healthy, type-II diabetic patients (T2D), and type-II diabetic patients with periodontitis (T2DP) samples. Significance was determined by Kruskal-Wallis analysis of variance (Kruskal and Wallis, 1952) for each comparison indicated, and Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) was applied to generate adjusted q-values. All data used were strictly filtered based on no-template control indexes. *q-value < 0.002 compared with healthy, **q-value < 0.0004 compared with healthy and with T2D. (C) Beta diversity Bray-Curtis distances. Pairwise PERMANOVA of each category versus each (group size of 3, n = 112) was performed in 999 permutations. ***q-value differs from "healthy" < 0.001. See also Figure S4.

microbiomes (Eren et al., 2014). Oligotyped "nodes" or representative sequences were aligned to both the Human Oral Microbiome (HOMD) (Escapa et al., 2018) and Ribosomal Database Project (RDP) (Cole et al., 2014) databases, and species were assigned based on 98% or higher sequence identity when comparing nodes to reference databases. We next compared species-assigned data between subject groups by linear discriminate analysis effect size measurements (LEfSe) (Segata et al., 2011). Using MED-analyzed data for healthy versus all subjects with T2D (Figure 3) we detected significant enrichments of some oral species (*Streptococcus cristatus, Neisseria flavescens*) and also some known reagent contaminants (*Ralstonia pick-etti, Afipia broomeae*) alongside other soil and freshwater microbes that we suspect are also contaminants (*Flectobacillus, Stenotrophomonas, Sphingobium, Arthrobacter*). Surprisingly, we observed *A. baumanii* significantly enriched in the T2D population as a whole. For MED analysis, reads assigned as *A. baumanii* were present from 0.01% to 7.3% of total reads in 8 and 5 subjects with T2D and T2DP, respectively. No reads assigned to *A. baumanii* were detected in any healthy subject. DADA2 amplicon sequence variants (ASVs) are similar to MED nodes, and we observed that one ASV node was a perfect match to the singular MED node assigned to *A. baumanii* based on 100% RDP alignment across its full sequence.

In our DADA2/QIIME2-based analysis we performed taxonomical assignment for each DADA2 ASV node aligned to either the Greengenes (DeSantis et al., 2006) (not shown) or SILVA database version 132 (Quast et al., 2013). Phylum-level taxonomic assignments of DADA2 data aligned to SILVA were used in Figure 2. ASVs are similar to MED nodes, and we observed that one ASV node was a perfect match to the singular MED node assigned to *A. baumanii* based on 100% RDP alignment across its full sequence. This ASV was assigned only to the *Acinetobacter* genus despite a 100% full-length match to *A. baumanii* sequence at the RDP database and the NCBI non-redundant database (not shown). Based on this, we manually assigned this ASV to *A. baumanii* and found that reads now assigned to *A. baumanii* were present from 0.01% to 9.1% of total reads in 8 and 5 subjects with T2D and T2DP, respectively, with no reads assigned to *A. baumanii* detected in any healthy subjects. This demonstrated agreement between both MED and
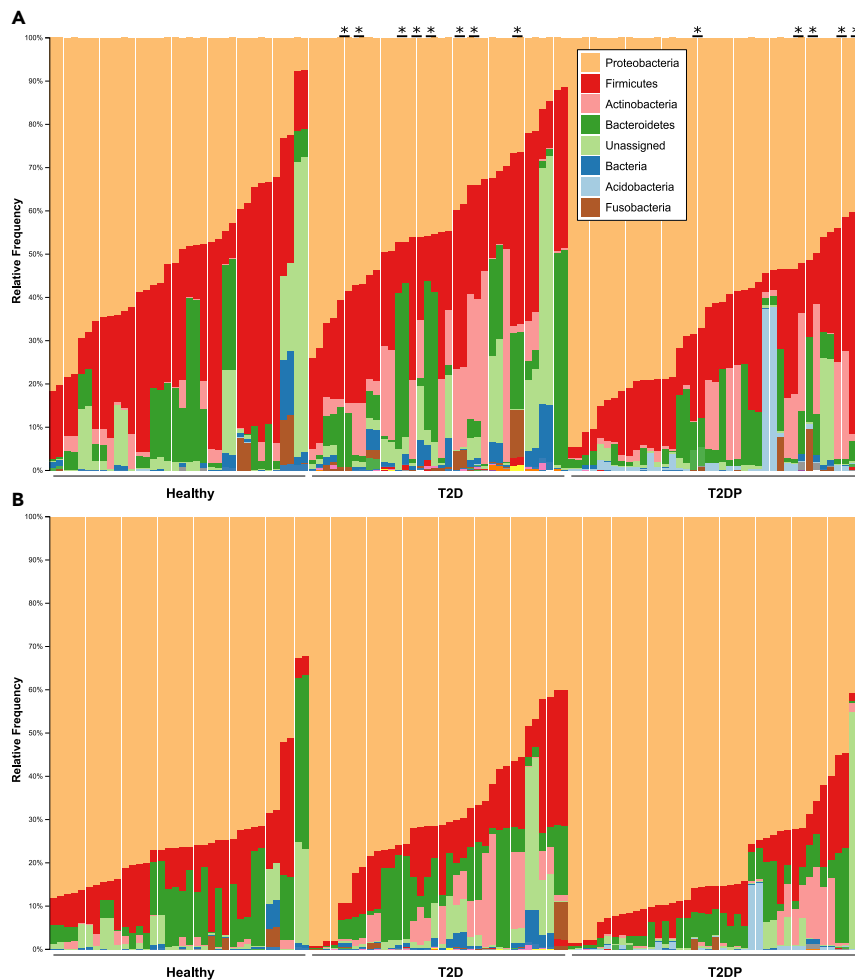
A

B

Figure 2. Phylum-level diversity changes between subject status

(A and B) DADA2-analyzed data were aligned to the SILVA database for taxonomical assignment via QIIME2 as described in Methods. Data from both sequencing runs were collapsed to phylum-level taxa and displayed for (A) strict filtered and (B) minimally filtered conditions. Asterisks on the top indicate run-run sample pairs that were positive for *A. baumanii*.

DADA2-based 16S data analysis. LEfSe analysis was again performed on healthy subjects versus all with T2D, as well as between all three subject groups, and demonstrated again that *A. baumanii* was signifi-cantly elevated among all T2D samples (Figure S5).

We next looked at differences in microbiome compositions across subjects by plotting Bray-Curtis distances via non-metric multidimensional scaling (NMDS) while distinguishing subject groups as well as presence or absence of ABC bacteria (Figure 4). Broadly, T2D and T2DP samples had less overlap in composition, whereas healthy samples intersected either group. ABC status did not seem to form a distinct microbiome cluster, suggesting that there was no unique microbiota composition for ABC-positive sub-jects apart from *A. baumanii* sequence detection.

Validating the presence of *A. baumanii* in subjects with T2D

The finding of ABC sequences was unexpected, as study participants were not hospitalized or under treat-ment for any infection(s), and we wished to confirm this result by other techniques. This was especially needed as the V1-V3 portion of the 16S rDNA sequence only differs between *A. baumanii* and *Acineto-bacter nosocomialis* by one nucleotide substitution. Using PCR primers for the 16S rDNA region that en-compasses nearly all differentiating 16S nucleotides between *Acinetobacter* species we generated ampli-cons from a portion of our ABC-positive samples alongside negative healthy and no-template control
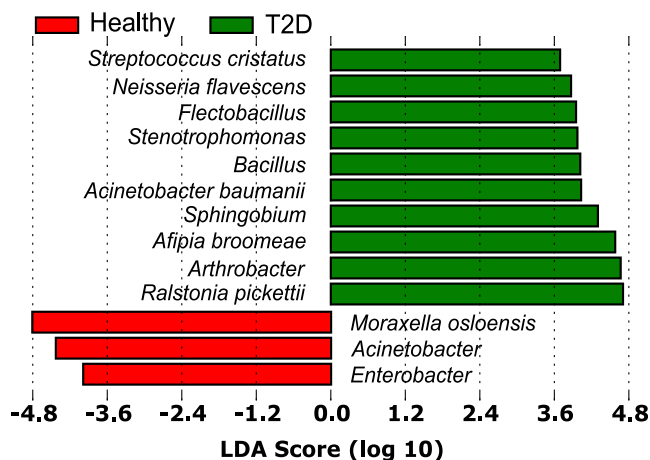
Figure 3. Healthy subjects versus all subjects with T2D reveal a potential enrichment of *A. baumanii*

Initial minimal entropy decomposition (MED) analysis of 16S data with taxonomy assigned based on RDP database alignment to generate species-level assignments was performed followed by linear discriminate analysis effect size quantification (LEfSe). This revealed enrichment of several species/genera in subjects with T2D relative to healthy. DADA2 analysis of the same dataset was aligned to the SILVA database and shows similar patterns of enrichment (see also Figure S5).

samples (Figure 5). PCR products were ligated into a TA vector, and individual colonies were propagated and subjected to Sanger sequencing of plasmid inserts. The 1242-bp sequences were then aligned to the NCBI reference RNA sequence database (refseq_rna). Each sequence (12 clones across 5 samples) was identical and matched the *A. baumanii* 16S sequence across its length. This result validated our initial 16S detection of putative *A. baumanii*.

Host cytokine responses to T2D and T2DP versus health

In addition to serum microbiota, we also quantified 20 different human cytokines in serum samples across all 3 patient groups (Figure 6). Based on relative abundance profiles, we observed that proinflammatory cytokines were significantly elevated in *A. baumannii*-positive samples highlighted in the box (ABC+). MCP-1 and IL-1β, classic proinflammatory cytokines, were increased in diabetic patients. Intriguingly, T2D ABC-negative subjects did not show increase of these markers. ABC+ subjects showed significantly higher expression levels of cytokines IL-1β, IL-6, IL-8, MCP-1, and IFN- γ when compared with ABC-negative groups (I and V, p < 0.01).

Host cytokine responses to specific microbiota

Based on the cytokine abundance data and earlier microbiome sequencing, we were able to determine if any specific taxa were significantly correlated with cytokine presence or absence in subjects with T2D (Figure 7). *A. baumanii* was significantly correlated with elevated amounts of IFN-α, IFN-γ, IL-12p70, IL-8, and TNF-α. ABC+ subjects presented cytokine correlations consistent with a profile of chronic inflammation. IL-8 is a chemokine that impacts neutrophil phenotype and function, and excessive amounts of this cytokine lead to chronic activation of phagocytes (Moore and Kunkel, 2019). Excessive production of TNF-α increases failure of resolution of inflammation on innate immune cells by controlling a resolution receptor (Freire and Van Dyke, 2013). Here, TNF-α showed a significant increase in ABC+ subjects, suggesting co-existence with ABC in the serum of patients with T2D. We also performed an identical analysis for healthy subjects and saw several genera or species with significant correlations (Figure S6). In healthy subjects unique profiles were found, correlated with lower levels of proinflammatory cytokines and co-clustered with ABC-negative samples.

Although low template concentration microbiome profiling presents many challenges, we were able to utilize numerous control steps to minimize the impact of contaminating taxa. We were also able to demonstrate that there were significant differences between subject groups that include taxa of likely biological origins, and also that specific cytokines significantly correlated with the presence of distinct taxa. Most surprisingly, we were able to detect *A. baumanii* and confirm its presence in serum samples of patients with
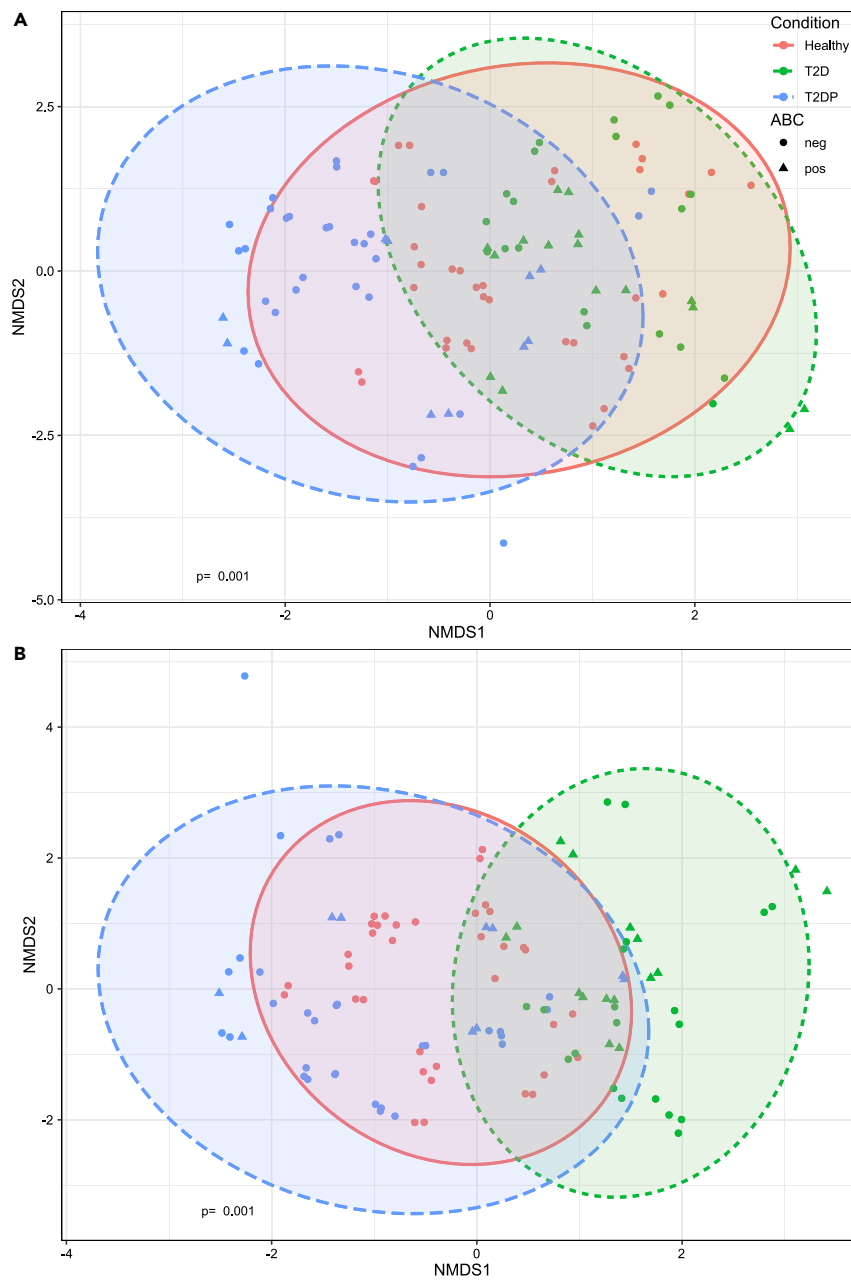
Figure 4. Beta diversity quantification between subject groups

(A and B) SILVA-assigned taxonomy of DADA2-analyzed 16S data was used to determine Bray-Curtis dissimilarity between samples for strictly filtered (A) and minimally filtered (B) data. Non-metric multidimensional scaling and statistical analysis for each subject group was performed in R using the *vegan* package as described in Methods. p-value of 0.001 determined by PERMANOVA test via the *adonis2* function.

T2D not known or currently diagnosed with any underlying infection. These data do not *per se* indicate active infection but could also indicate colonization or transient exposure to *A. baumanii* for these subjects compared with healthy subjects.

Discussion

Issues with amplification-based 16S studies from low template concentrations are well known (Sinha et al., 2015; Kim et al., 2017; Pollock et al., 2018). We began this study as a *post hoc* analysis of existing serum samples from a
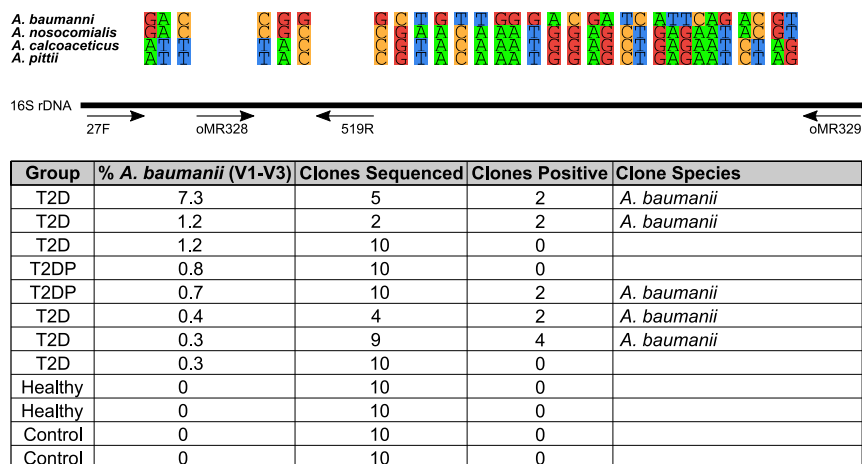
Group	% *A. baumanii* (V1-V3)	Clones Sequenced	Clones Positive	Clone Species
T2D	7.3	5	2	*A. baumanii*
T2D	1.2	2	2	*A. baumanii*
T2D	1.2	10	0	
T2DP	0.8	10	0	
T2DP	0.7	10	2	*A. baumanii*
T2D	0.4	4	2	*A. baumanii*
T2D	0.3	9	4	*A. baumanii*
T2D	0.3	10	0	
Healthy	0	10	0	
Healthy	0	10	0	
Control	0	10	0	
Control	0	10	0	

Figure 5. Clone library design to verify ABC complex member identity

Amplification with oMR328-329 primers produced an amplicon between 120 and 1306 bp of the *A. baumanii* 16S rDNA sequence. This allowed for Sanger sequencing of information-rich nucleotides indicated above allowing for species differentiation beyond initial V1-V3 sequencing with the 27F-519R primers. The table beneath indicated the total number of individual clones sequenced per sample and which species they aligned to with 100% identity in the NCBI non-redundant database.

previous bank of anonymized specimens (Freire et al., 2017). As sample volumes of serum were mostly limited, we relied on amplification of 16S rDNA, using V1-V3 region primers to improve the likelihood of identifying oral taxa (Eren et al., 2014) that we hypothesized to exist in the diabetic with periodontitis part of our cohort. To minimize effects of amplification, we performed multiple high-throughput sequencing runs of amplified libraries to increase sequence depth as opposed to further amplification and using a higher capacity sequencing platform. Additionally, we also included numerous no-template controls throughout sample preparation and purification to identify as many outside contaminants as we could (Kennedy et al., 2014; Salter et al., 2014; Kim et al., 2017). As a further control, we carried out amplification of mock community libraries (Figure S1) to determine if amplification bias or infidelity affected taxonomic assignment. We also used two separate methods for correction of Illumina-sequenced amplicon errors, DADA2 (Callahan et al., 2016) and minimal entropy decomposition/oligotyping (MED) (Eren et al., 2015), as well as comparing our data from multiple ribosomal databases for taxonomic assignment (DeSantis et al., 2006; Quast et al., 2013; Cole et al., 2014). In addition to Illumina sequencing, we performed a separate amplification of 16S rDNA followed by Sanger sequencing to independently confirm the identity of putative *A. baumanii* sequence presence in a subset of our diabetic samples (Figure 5). Thus, we have made great efforts to validate our findings as far as is feasible and have taken care not to overinterpret data presented here.

We initially hypothesized that oral taxa would be enriched in the bloodstream of subjects with T2DP, and potentially all subjects with T2D, compared with healthy subjects. Our microbiome data revealed changes in composition between our three subject groups (Figures 1, 2, 3, 4, S4, and S5). However, there was only enrichment of few oral taxa when healthy subjects were compared with all subjects with T2D and not with subjects with T2DP alone, specifically *S. cristatus* and *N. flavescens* (Figure 3), which are not associated with periodontitis. Their presence here could indicate further access to the bloodstream by oral microbes in the T2D cohort, although this requires further verification. Given the limited template available for each sample, we cannot rule out that other oral taxa are not elevated in subjects with T2D/P, but more rigorous testing of that hypothesis would require larger serum volumes and using a longitudinal approach. By far the most surprising element of this dataset was the detection of *A. baumanii*- (Figures 3 and S5) assigned reads unique to 13 subjects with T2DP and not present in any control or healthy subject samples.

A. baumanii represents a considerable risk to subjects with T2D including higher mortality rates (Leão et al., 2016) and a higher risk of infection in burn wounds, as well as DFIs (Furniss et al., 2005; Castellanos et al., 2019; Henig et al., 2020). To confirm our V1-V3 amplicon data and to determine whether or not these were *A. baumanii* or another ABC species, we used targeted near-full-length 16S rDNA primers that allowed us to sequence the majority of differentiating nucleotides necessary for ribotyping. Sequencing individual clones of these amplicons
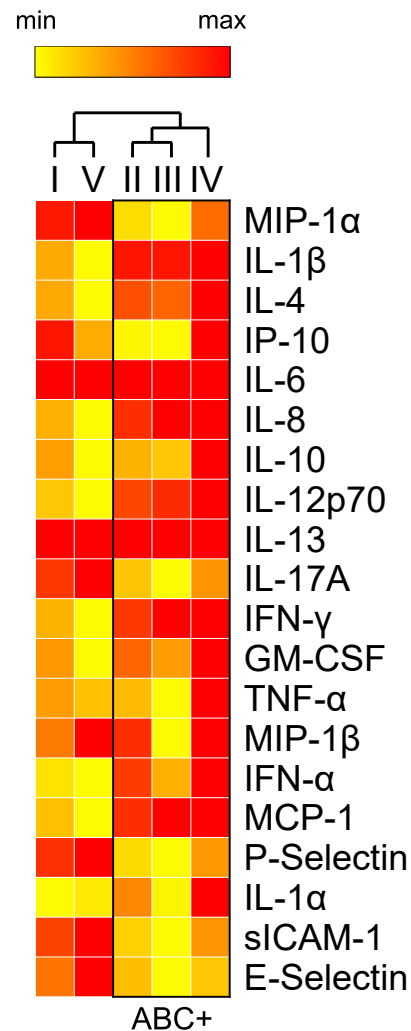
Figure 6. Cytokine profile quantification reveals distinct differences between subject groupings and *A. baumanii* (ABC) presence

Heatmap showing immune mediator concentrations derived from serum of diabetic and healthy samples were compared to understand the role of ABC in each sample group. (I) Diabetic/Healthy, (II) All ABC+/All ABC-, (III) ABC+ Diabetic/ABC-Diabetic, (IV) ABC+ Diabetic/Healthy, (V) ABC- Diabetic/Healthy. Data are clustered according to comparison groups (red, highest expression; yellow, lowest expression). Hierarchical clustering was employed by Morpheus from the Broad Institute (Morpheus, https://software.broadinstitute.org/morpheus). Observations from hierarchical clustering are shown in a tree, with ABC groups highlighted in the box. ABC+ groups (II, III, and IV) showed significantly different ($p < 0.01$) levels of IL-1β, IL-6, IL-8, MCP-1, IFN-γ. *p value <0.01 between groups via unpaired Student's t test (red, highest expression; yellow, lowest expression).

revealed 100% matches to *A. baumanii,* and no clones (n = 50) were *A. baumanii* positive from indicated control samples (Figure 5). This result was surprising, as none of the subjects in our T2D/P cohort were currently under inpatient treatment or had any indication of underlying infection (apart from periodontitis). Given the methodology here, neither can we speculate on the true infection status of these individuals nor can we be certain if the *A. baumanii* present were circulating in the bloodstream or were on the skin and mixed with the sample during a blood draw. No matter the route of entry, we were able to determine significant cytokine profile changes in T2DP *A. baumanii*-positive subjects, as well as broader inflammatory changes in cytokine profiles between subjects with T2DP and T2D and healthy subjects (Figure 6).

Cytokine dysregulation can impact the host locally and systemically, making the subject more prone to severe infections and increased tissue damage (Tisoncik et al., 2012). Individuals who have T2D present
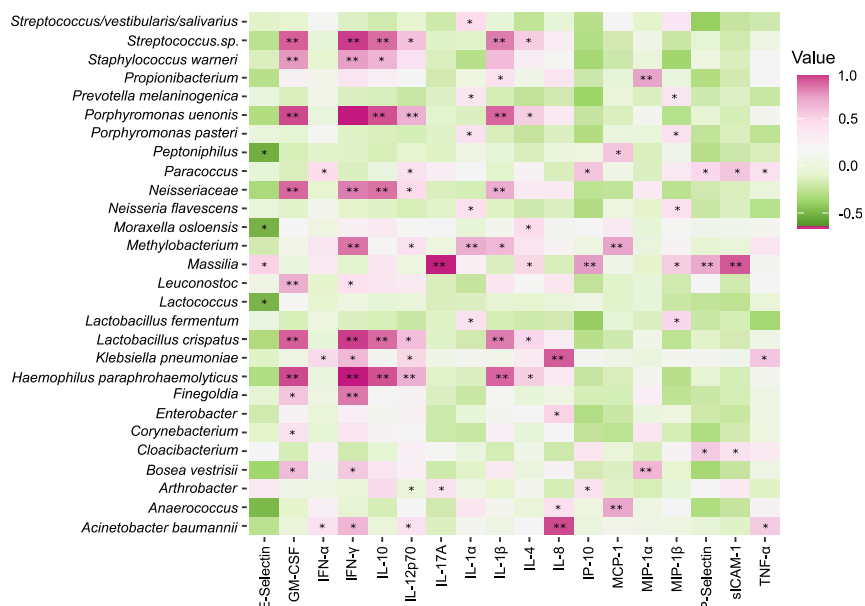
Figure 7. Host-microbial correlation-resolved type-II diabetic associations with inflammatory cytokines

HOMD/RDP assigned taxonomy of MED-analyzed 16S data for both T2D and T2DP samples and cytokine concentrations were analyzed via Pearson correlation coefficient in R using the *rcorr* function. Significance was determined using the asymptotic p values generated by *rcorr* with *p value <0.05, **p value <0.01. Data are strictly filtered with taxa present in no-template controls subtracted. See also Figures S6, S8, and S9.

an increased metabolic burden, while often comorbid periodontal disease promotes microbial dysbiosis, with both diseases resulting in a chronic state of inflammation. In the present study, *A. baumanii* was only found in the serum of diabetic subjects, where an increase in proinflammatory cytokines was also observed (Figures 6 and 7). We utilized an unbiased panel of 20 human cytokines to survey specific immune responses to T2D and T2DP compared with those of healthy individuals. Although there are complications presented by low template sizes in our samples, cytokine profiling from these same volumes were well within desired assay volumes. As mediators of inflammation, cytokine production feeds forward a cascade of signals that can impact tissue, organ, and overall host immunometabolism. In T2D, IL-1β, TNF-α, MCP-1, IL-6, IL-8, and IFN-γ were significantly elevated in ABC+ subjects and not in ABC-negative subjects. Of these proinflammatory cytokines, IL-1β, TNF-α, and IL-8 suppression has been investigated as therapeutic targets for clinical management of diabetes and associated chronic lesions (Peiró et al., 2017; Feng et al., 2018). We previously observed increased TNF-α levels in cell cultured neutrophils from subjects with T2D relative to cells derived from healthy subjects (Kleinstein et al., 2020a). Co-occurrence of bacteria in the serum and cytokines also differed among controls. In the current study, co-occurrence among cytokines was more evident in diabetic subjects (100 significant co-occurrences) versus healthy individuals (30 significant co-occurrences) (Figures 7 and S6). *A baumannii* especially correlated with neutrophil inflammatory signaling cytokine IL-8, which was not observed in healthy subjects. We have previously shown differences in gene expression in neutrophils of individuals with T2D, which are primed to mount an aberrant inflammatory response when compared with neutrophils of healthy individuals (Kleinstein et al., 2020a), consistent with production of inflammatory cytokines observed here. In contrast, IL-17α, P-selectin, and E-selectin levels were increased in diabetic subjects who were ABC-negative compared with ABC+ diabetic subjects. IL-4 and IL-10 did not co-occur with *A. baumanii* in any of the healthy versus diabetic groups. The cytokine results provide an initial glimpse into the host response to chronic inflammatory diseases, T2DP with and without the presence of *A. baumannii*, which may warrant further investigation in larger studies.

In conclusion, this study has revealed modest serum microbiome compositional changes and significant cytokine profile changes between subjects with T2D with or without periodontitis versus non-T2D individuals, revealing an impaired host response when *A. baumanii* was present. We also present here the potential for lurking *A. baumanii* colonization in asymptomatic subjects with T2D, possibly to

a much larger extent than previously reported. In our opinion, these data present a strong justification for further monitoring of individuals with T2D for *A. baumanii* colonization and support the need for a more robust study to characterize the potentially greater spread of *A. baumanii* among individuals with T2D.

Limitations of the study

Limitations of this study primarily include small template volumes available for serum samples in this *post hoc* study. Blood and sera microbiome studies will always be more complex due to low bacterial template concentrations inherent to these types of sample. Although we have made utmost efforts to validate our main findings regarding *A. baumanii* presence, there are likely more interesting aspects to our study cohort microbiota that have been missed. These and other minor limitations have been indicated in the article.

Resource availability

Lead contact

Further information and requests for resources, data, code, and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Matthew Ramsey (mramsey@uri.edu).

Materials availability

This study did not generate new unique reagents or genetic constructs.

Data and code availability

All data is available at NIH – SRA Bioproject: PRJNA664044. All other code used for data analysis has been provided in the Transparent Methods section (see Scripts) in the Supplemental Information.

Methods

All methods can be found in the accompanying Transparent Methods supplemental file.

Supplemental information

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101941.

Author contributions

D.P., B.H., and S.E.K. performed the work. D.P., S.E.K., M.F., and M.R. wrote and edited the manuscript. M.F., H.H., and M.R. provided reagents and samples. D.P., R.E., S.E.K., M.F., and M.R. designed the experiments. M.F. and M.R. secured funding.

Declaration of interests

The authors declare no competing interests.

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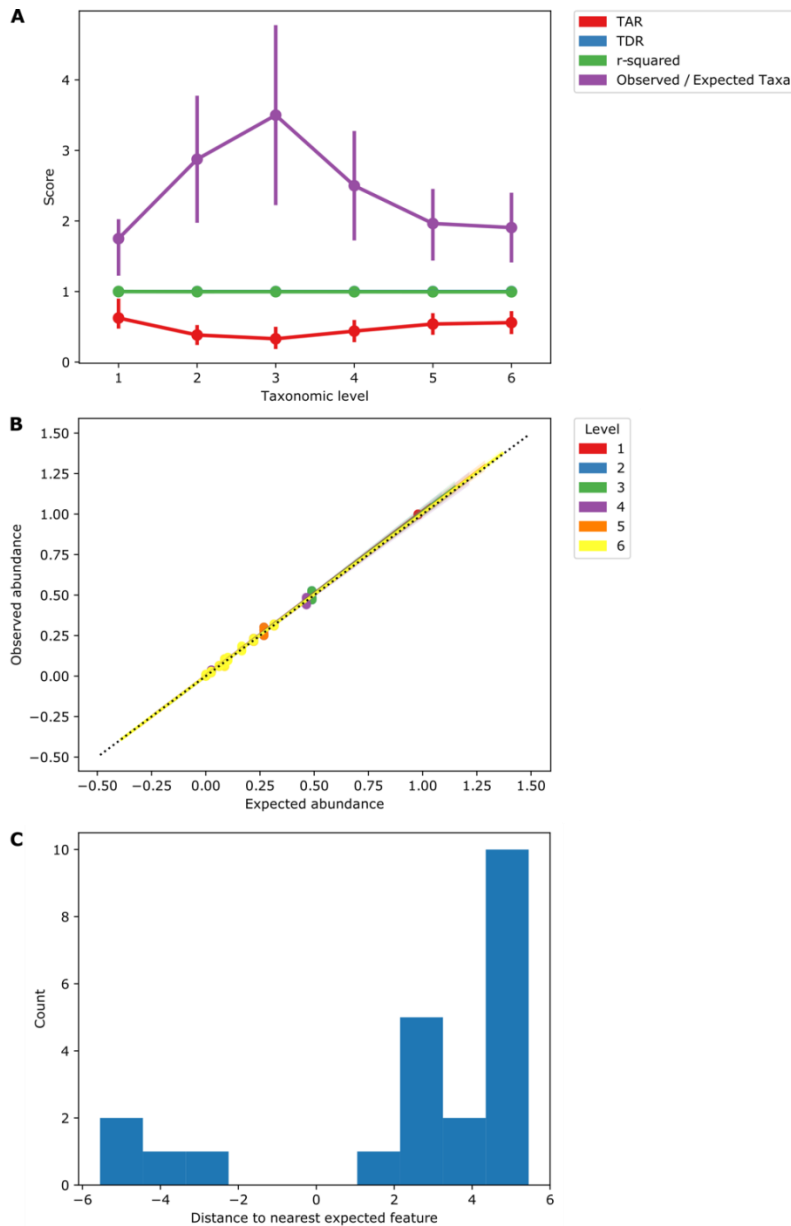
Supplemental Information

Impaired host response and the presence

of *Acinetobacter baumannii* in the serum

microbiome of type-II diabetic patients

Dasith Perera, Sarah E. Kleinstein, Benjamin Hanson, Hatice Hasturk, Ryan Eveloff, Marcelo Freire, and Matthew Ramsey

Supplemental Information

Supplemental Figures and Legends

Figure S1. Mock library composition evaluation. Related to Figure 1. A Zymogen mock community library was used for sequence library construction at 10 and 1 ng in duplicate between both sequencing runs. QIIME2 / DADA2 unfiltered data was given taxonomical assignment via SILVA and assessed via the "q2-quality-control" plugin. (A) Taxon accuracy rate (TAR), taxon detection rate (TDR) and linear regression scores (r-squared) are plotted at each taxonomic level. (B) Expected vs observed abundance of each species in the mock community is plotted at each taxonomic level. (C) Distance between false positive observations vs nearest expected feature. Misclassifications were unique to environmental contaminant taxa and no false negative features were observed (not shown).

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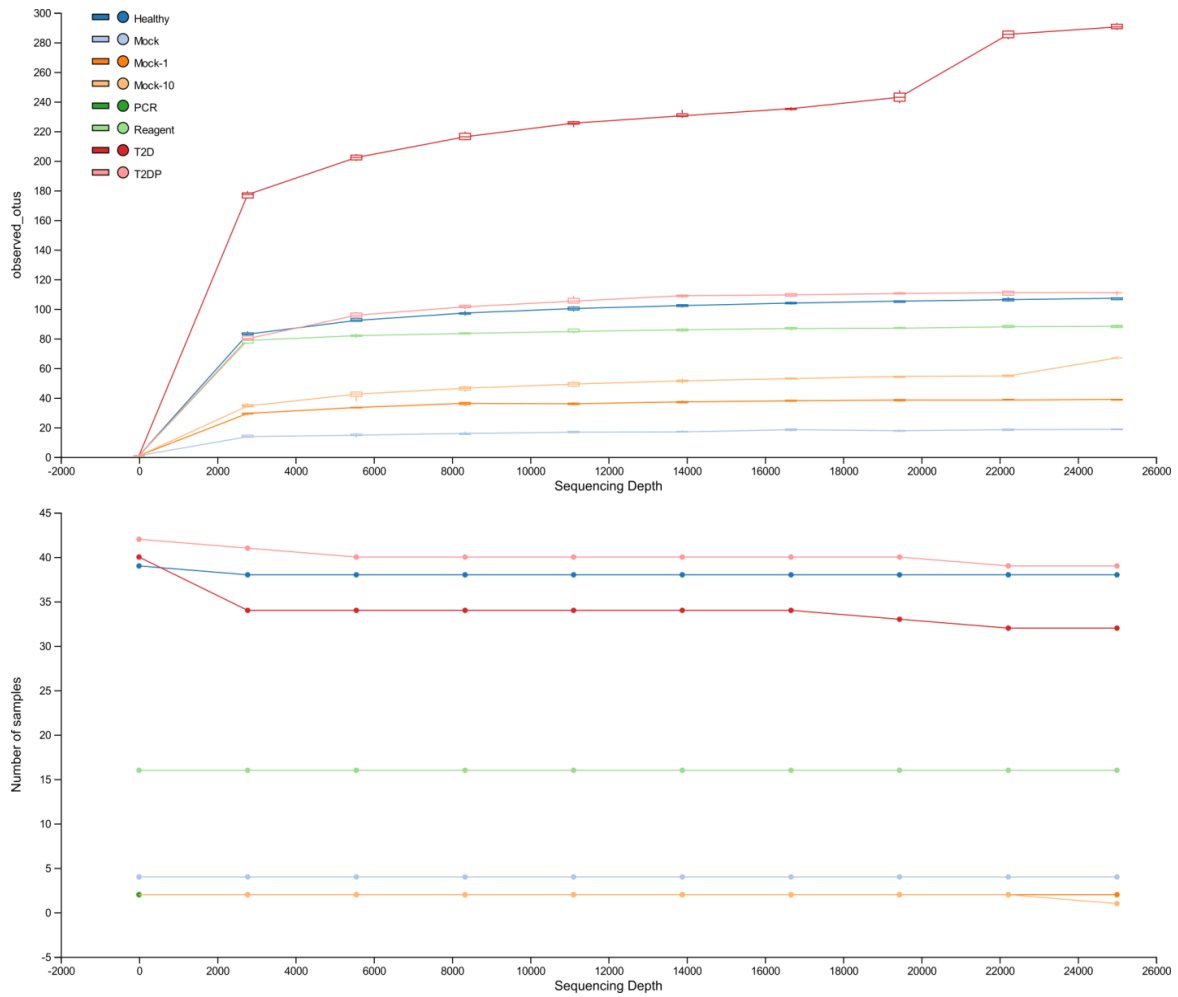
Figure S2. Alpha-diversity at different rarefaction levels. Related to Figure 1. Total observed OTUs (here ASVs) are plotted (A) vs sequencing depth and each sample that can accommodate that sequencing depth is plotted in (B).

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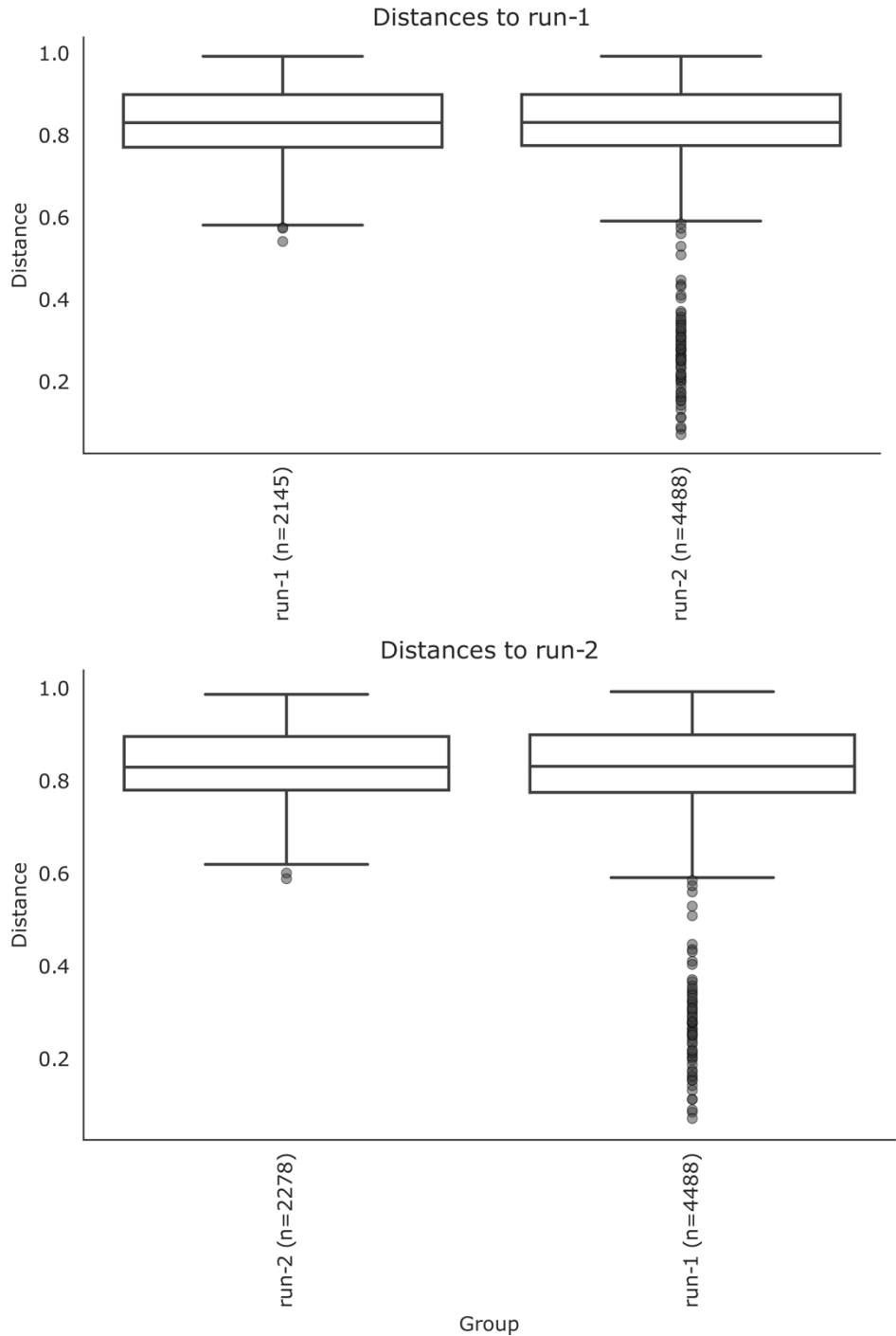
Figure S3. Run to Run variability quantification. Related to Figure 1. A Run vs Run Beta
diversity comparison was performed for unfiltered data by comparing unweighted Unifrac
distances as described in the methods. Pairwise PERMANOVA of Run-1 vs Run-2 (group size
of 2, sample size of n=134 total indexes) was performed in 999 permutations resulting in a
pseudo-F value of 0.567, p-value of 0.999 and corrected q-value of 0.999. No significant
differences were observed between each run.

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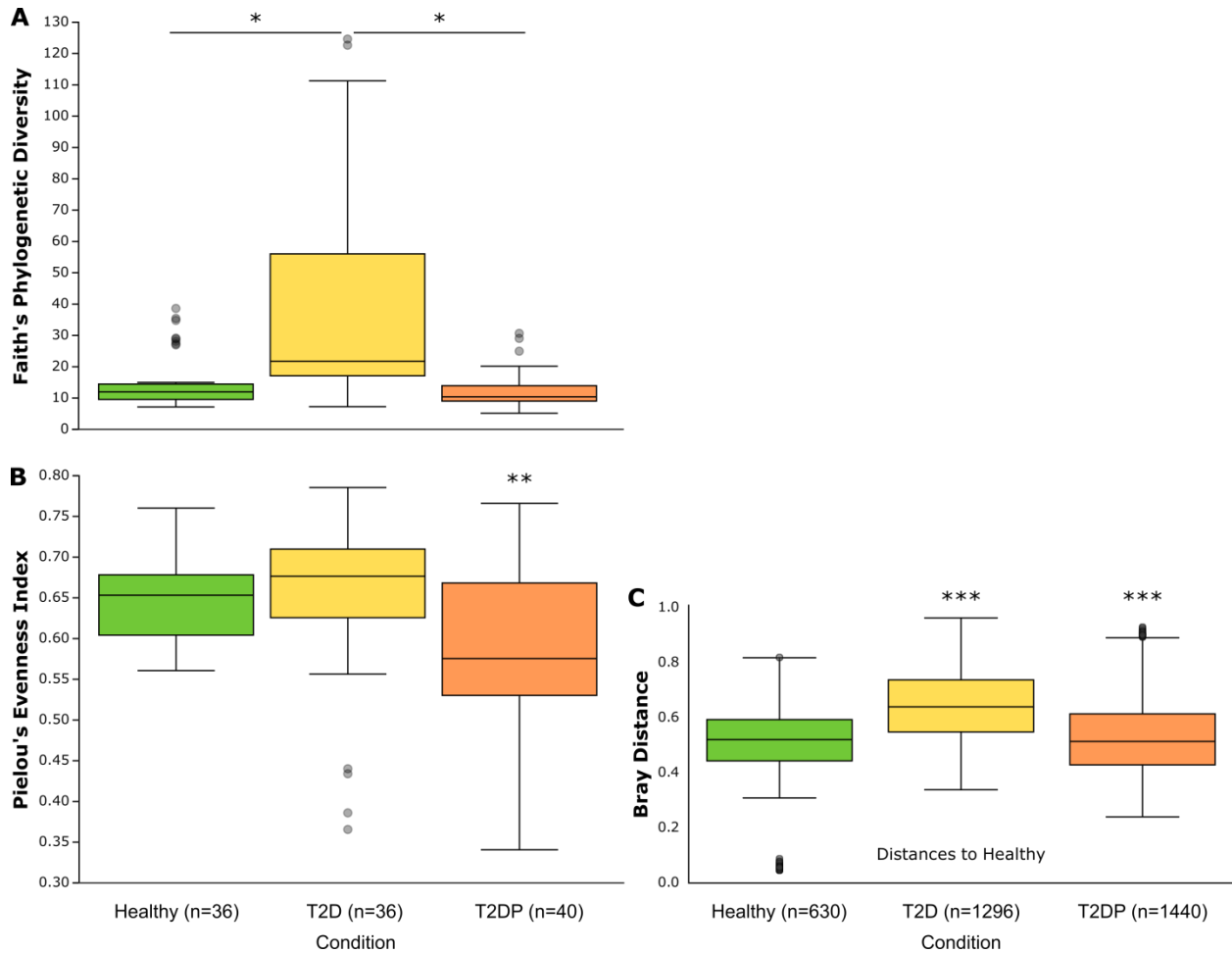
Figure S4. 16S Alpha and Beta diversity of serum microbiomes changes with subject status. Related to Figure 1. Species richness is displayed based on Faith's phylogenetic diversity (Faith, 1992) (A) and species evenness based on Pielou's evenness index (Pielou, 1966) (B) for healthy, type-II diabetic (T2D) and type-II diabetics with periodontitis (T2DP) samples. Each graph used minimally filtered data for analysis. Significance was determined by Kruskal-Wallis analysis of variance (Kruskal and Wallis, 1952) for each comparison indicated and Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) was applied to generate adjusted q-values. * indicates q-value < 2e-3 vs healthy, ** indicates q-value < 0.024 vs healthy, (C) Beta-diversity Bray Curtis distances. Pairwise PERMANOVA of each category vs each (group size of 3, n=112) was performed in 999 permutations. *** indicates q-value differs from healthy < 0.001.

39

A Legend:
- Healthy
- T2D

a: D_5__Granulicella
b: D_4__Acidobacteriaceae_Subgroup1_
c: D_3__Acidobacteriales
d: D_5__Lawsonella
e: D_4__Intrasporangiaceae
f: D_5__unculturedbacterium
g: D_4__Microbacteriaceae
h: D_5__Arthrobacter
i: D_5__Rothia
j: D_3__Micrococcales
k: D_5__Cutibacterium
l: D_4__Propionibacteriaceae
m: D_3__Propionibacteriales
n: D_5__Prevotella7
o: D_4__Prevotellaceae
p: D_5__Flectobacillus
q: D_4__Spirosomaceae
r: D_3__Cytophagales
s: D_5__Bacillus
t: D_4__Bacillaceae
u: D_5__Planococcus
v: D_5__Jeotgalicoccus
w: D_5__Staphylococcus
x: D_4__Staphylococcaceae
y: D_5__Lactococcus
z: D_4__Streptococcaceae
a0: D_3__Lactobacillales
a1: D_5__Veillonella
a2: D_5__Acidisphaera
a3: D_4__Acetobacteraceae
a4: D_3__Acetobacterales
a5: D_5__Niveispirillum
a6: D_5__Caulobacter
a7: D_3__Bdellovibrionales
a8: D_5__unculturedRoseobactersp_
a9: D_4__0319_6G20
b0: D_5__Herbaspirillum
b1: D_5__Schlegelella
b2: D_5__Neisseria
b3: D_5__Enterobacter
b4: D_5__Acinetobacter
b5: D_4__Moraxellaceae
b6: D_3__Pseudomonadales
b7: D_5__Stenotrophomonas
b8: __
b9: D_4__Xanthomonadaceae
c0: D_3__Xanthomonadales

B Legend:
- Healthy
- T2D

C Legend:
- Healthy
- T2D
- T2DP

40

Figure S5. LEfSe Comparisons reveal taxonomical differences between subject groups. Related to Figure 3. (A) LEfSe Cladogram plots (Segata *et al.*, 2011) reveal general taxonomic shifts compared to (B) specifically enriched genera or species in all diabetics vs healthy and (C) compared across all 3 subject groups. Data was generated from DADA2 / QIIME2 analyzed data aligned to the SILVA database that was then filtered for contaminating taxa abundant in no-template control indexes. *Pseudomonas* genus / species level results were also removed for clarity.

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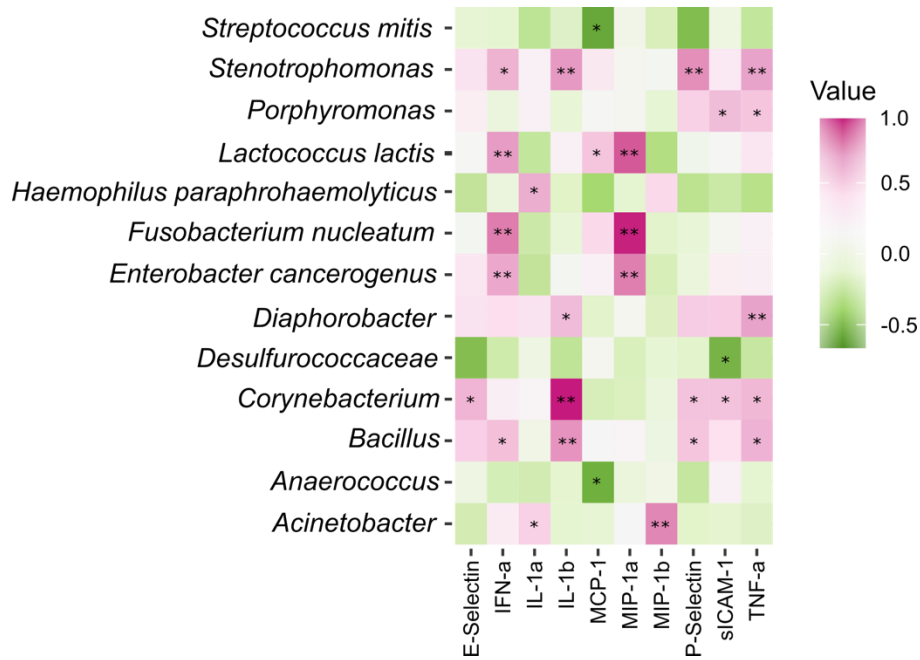
Figure S6. Cytokine correlation with specific taxa in healthy subjects. Related to Figure 7. HOMD/RDP assigned taxonomy of MED analyzed 16S data for healthy samples and cytokine concentrations were analyzed via Pearson correlation coefficient in R using the *rcorr* function. Significance was determined using the asymptotic p-values generated by *rcorr* with * = p-value <0.05 ** = p-value <0.01. Data are strictly filtered with taxa present in no-template controls subtracted.

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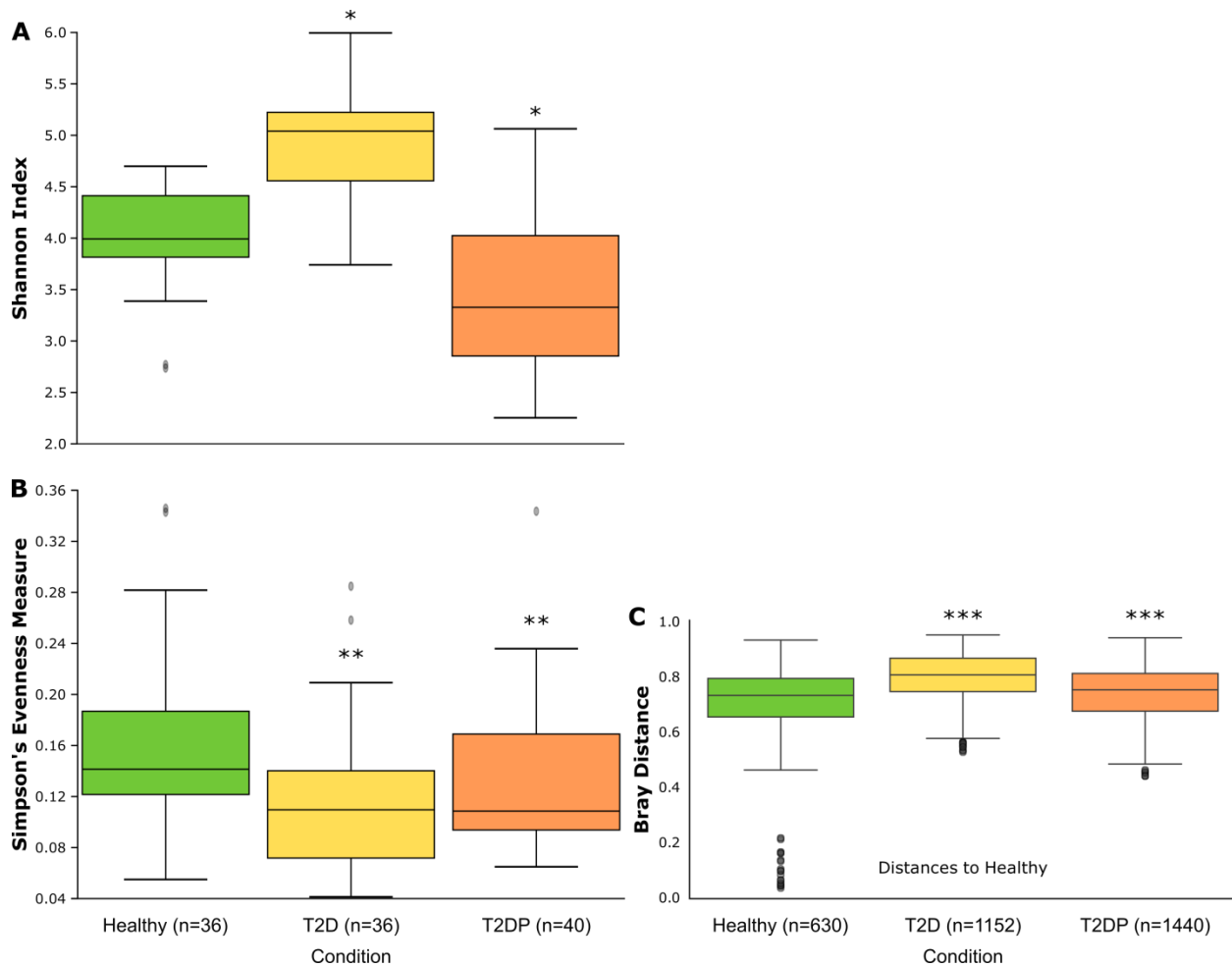
Figure S7. 16S Alpha and Beta diversity of serum microbiomes changes with subject status. Related to Figure 1. Alpha diversity is displayed based on Shannon's index (Shannon and Weaver, 1975) (A) and species evenness based on Simpson's evenness measure (Simpson, 1949)) (B) for healthy, type-II diabetic (T2D) and type-II diabetics with periodontitis (T2DP) samples. Each graph used minimally filtered data for analysis. Significance was determined by Kruskal-Wallis analysis of variance (Kruskal and Wallis, 1952) for each comparison indicated and Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) was applied to generate adjusted q-values. * indicates q-value < 1e-5, ** indicates q-value < 0.007, (C) Beta-diversity unweighted Unifrac distances. Pairwise PERMANOVA of each category vs each (group size of 3, n=112) was performed in 999 permutations. *** indicates q-value differs from 'healthy' < 0.02.

72
Figure S8. Host-microbial correlation resolved type-II diabetic associations with inflammatory cytokines. Related to Figure 7. SILVA assigned taxonomy of DADA2 analyzed 16S data for both T2D and T2DP samples and cytokine concentrations were analyzed via Pearson correlation coefficient in R using the *rcorr* function. Significance was determined using the asymptotic p-values generated by *rcorr* with * = p-value <0.05 ** = p-value <0.01. Data are strictly filtered with taxa present in no-template controls subtracted.
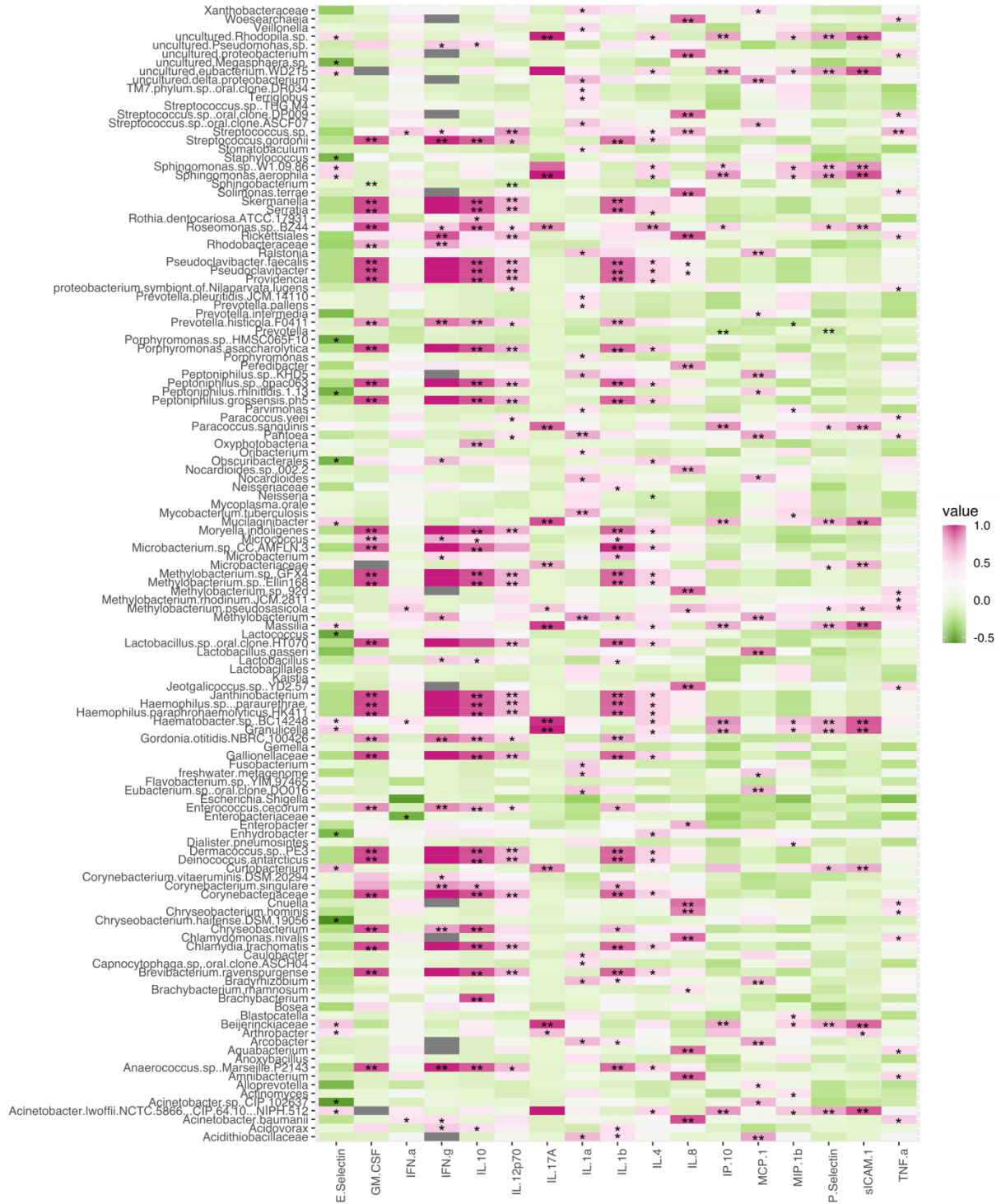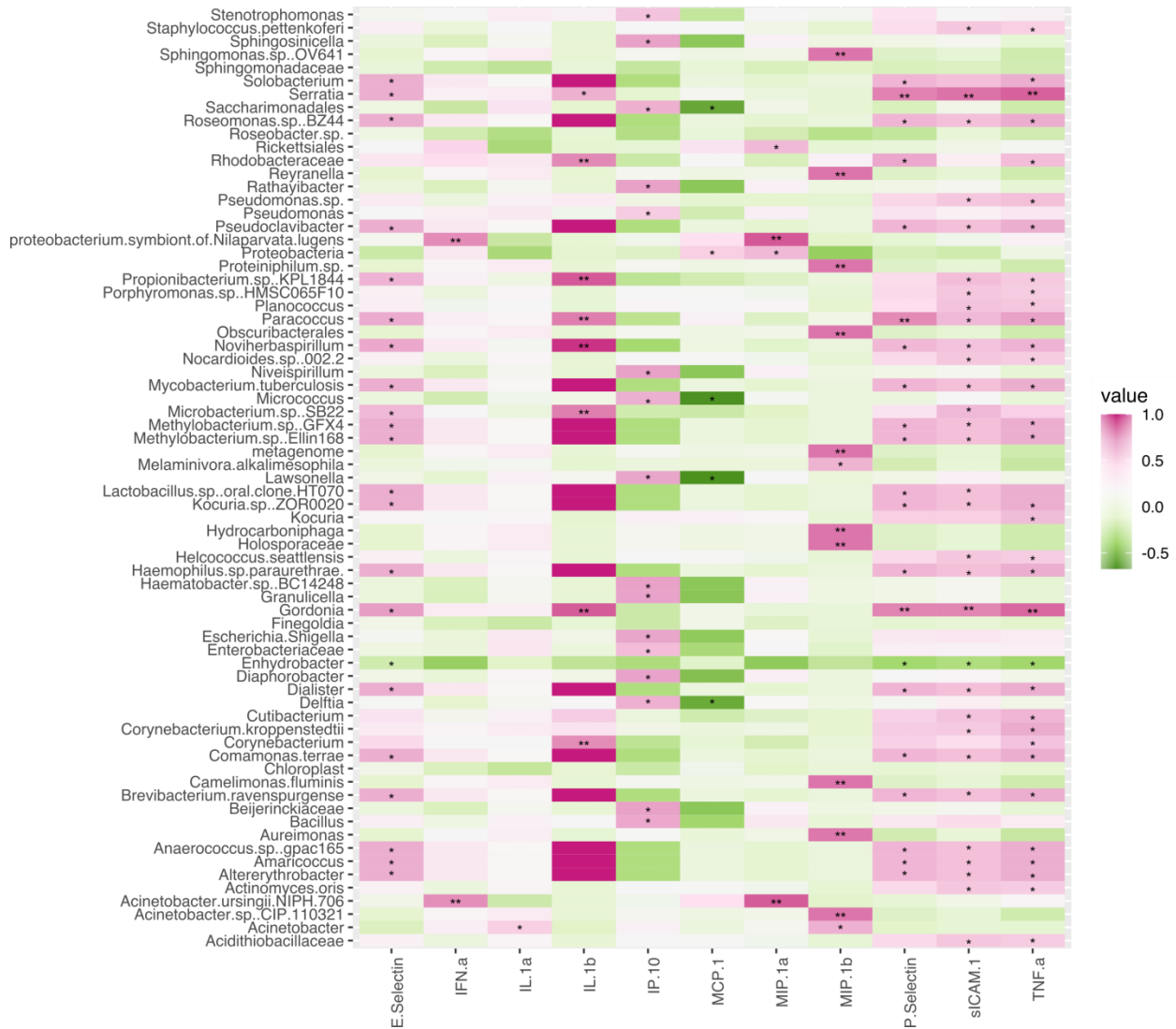
79
Figure S9. Cytokine correlation with specific taxa in healthy subjects. Related to Figure 7.
SILVA assigned taxonomy of DADA2 analyzed 16S data for healthy samples and cytokine
concentrations were analyzed via Pearson correlation coefficient in R using the *rcorr* function.
Significance was determined using the asymptotic p-values generated by *rcorr* with * = p-value
<0.05 ** = p-value <0.01. Data are strictly filtered with taxa present in no-template controls
subtracted.
86

Transparent Methods

Subject recruitment, sampling and storage

Subject recruitment has been described previously (Freire *et al.*, 2017). Peripheral venous blood (∼60 ml) was collected from patients diagnosed with T2D and from healthy nondiabetic controls. Blood samples were collected and centrifuged at 2300 rpm, and serum was isolated and frozen at -80°C until analysis under IRB protocol #13-07. All subjects gave signed informed consent prior to study evaluations. Clinical periodontal data and peripheral venous blood were collected. The diagnosis of T2D was made by the subject's primary care physician following American Association of Diabetes guidelines (American Diabetes Association, 2015). Information was collected on subject demographics (age, gender, self-reported ethnicity, and self-reported smoking status), body-mass index (BMI; kg/m2), blood total cholesterol, blood glucose (point-of-care), percent hemoglobin A1C (HbA1c), and periodontal condition (Armitage, 1999). HbA1c was used to determine the level of glycemic control for diabetic subjects. One T2D individual lacked HbA1c measurements but fit based on all other diagnostic criteria (blood glucose >200 mg/dl) as well as cytokine profiles in accord with other T2D individuals. Neutrophil and monocyte cell counts were determined by lab assay (described below). Individuals were excluded if they were taking insulin sensitizers, nonsteroidal anti-inflammatory drugs, or antimicrobials within 3 months of study initiation. Smoking status was defined by CDC NHIS terms (https://www.cdc.gov/nchs/nhis/tobacco/tobacco_glossary.htm#:~:text=Former%20smoker%3A%20An%20adult%20who,in%20his%20or%20her%20lifetime). Site collection before blood draw: Samples were drawn in clinical phlebotomy settings. From all serum sampled for this microbiome study, a total of 81 subjects (N=24 healthy, N=57 T2D) were included for analysis, all of whom were unrelated and over 18 years of age (range: 28-79 years of age).

Sample preparation for 16S analysis

DNA Extraction

Frozen serum from N=81 samples (N=24 healthy, N=32 T2D and N=25 T2DP) were thawed on ice and aliquots were separated for DNA extraction. DNA was extracted using the Epicentre MasterPure Complete DNA and RNA Purification kit (Lucigen, WI, USA). Extraction was carried out using the manufacturer's instructions, with modifications that enabled bead beating. Briefly, 200µL of Tissue and Cell Lysis Solution (2x), 100µL of nuclease free water and 2µL of Proteinase K were added to Lysing matrix B (LMB) tubes (MP Biomedicals, Santa Ana, CA). Thereafter 100µL of serum was added to the tubes and placed in a Beadbeater (Biospec) for 30 seconds, then placed on ice for 3 minutes and then repeated. Each round of DNA extractions included a no template control, which consisted of 100µL nuclease free water instead of serum. The samples were then incubated and extracted as described in the manufacturer's instructions. Total DNA was precipitated using the manufacturer's instructions, however 300 µL of MPC Protein Precipitation Reagent was used to accommodate the increased volume. DNA was eluted in 50µL of nuclease free water.

16s rDNA primers 27F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATYMTGGCTCAG) and 519R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGWATTACCGCGGCKGCTG) (Stackebrandt and Goodfellow, 1991; Turner *et al.*, 1999) were used to amplify the V1-V3 regions of 16s rDNA in a 50 µL reaction using 2x Q5 HiFi mastermix (New England Biolabs, Ipswitch, MA) and 23 µL of extracted DNA (35 cycles).

137
Illumina MiSeq Library preparation

139
After the first round of PCR was carried out it was cleaned with Ampure XP beads (Beckman Coulter, Pasadena, CA) and then visualized by agarose gel electrophoresis. Full indices and adapters were added using the Illumina Nextera Index Kit (Illumina, San Diego, CA) by running the second round of PCR (50 ng of template DNA, 5 cycles) in 2x Phusion HF Master Mix (New England Biolabs, Ipswitch, MA). PCR were then cleaned with Ampure XP beads and analyzed by agarose gel electrophoresis and using the Agilent BioAnalyzer DNA1000 chip (Agilent Technologies, Santa Clara, CA). Samples were then quantified and normalized prior to pooling using a Qubit fluorimeter (Invitrogen, Carlsbad, CA). The final pooled library was quantified with the KAPA Biosystems Illumina Kit (KAPA Biosystems, Woburn, MA) via qPCR in a Roche LightCycler480. Samples were sequenced (2x250 bp paired-end) on an Illumina MiSeq (Illumina, San Diego, CA) at the Rhode Island Genomics and Sequencing Center (Kingston, RI).

151
Mock Community Standards Preparation

153
In order to quantify potential bias due to low template concentrations, PCR amplification, and contamination, we performed mock library assemblies with commercially available bacterial genomic DNA templates (Zymo Research #D6305). We amplified 2 libraries using 10 and 1 ng total starting templates in both sequencing runs for a total of 4 mock libraries synthesized.Total DNA species composition is provided in the manufacturer's instructions.

158
16S clone library resequencing

159
Primers were made to amplify regions of 16s rDNA that enable differentiation of the Acinetobacter species. Primers used were
oMR328 (TAGCGGCGGACGGGTGAGTAATGCTTA) and
oMR329 (TTCCGACTTCATGGAGTCGAGTTGCAGAC). 50 µL reactions in 2x Q5 mastermix (New England Biolabs, Ipswitch, MA) were run for 35 cycles using 23 µL of DNA extracted from serum as the template. The products were then purified using the Qiagen PCR purification kit according to the manufacturer's instructions. Gotaq 2x mastermix (Promega, Madison, WI) was used to generate 'A' overhangs via PCR for 5 cycles using 10 µL of purified DNA as the template. 4µL of the resulting PCR products were then used to generate clones using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions which was modified to use 0.5µL of TOPO vector. Clones were then transformed into NEB5α cells using the manufacturer's instructions and plated on LB plates supplemented with 0.1mM IPTG and 40µg/mL Kanamycin. Additionally, 40µl of 40mg/mL of X-gal was spread onto each plate prior to plating to enable blue-white screening. The plates were incubated for 24 hours at 37°C. White colonies were inoculated into liquid cultures of LB supplemented with 40µg/mL kanamycin. Plasmids were extracted using QIAprep Spin Miniprep kit (Qiagen, Venlo, Netherlands). Inserts were sequenced using the M13 Forward (GTAAAACGACGGCCAGTG) and M13 Reverse (CACAGGAAACAGCTATGACC) primers on an Applied Biosystems 3500xl using the "BigDye" Terminator v3.1 Cycle sequencing kit. Sequences were analyzed using NCBI BLAST (NCBI Resource Coordinators, 2018). A maximum of 10 colonies were screened for each sample or until a sequence matched a species belonging to the *Acinetobacter baumanii- calcoaceticus* complex.

182
Cytokine Quantification

184

185 Frozen (-80°C) subject serum was brought up to room temperature and assayed using the
186 Invitrogen human inflammation 20-plex ProcartaPlex cytokine panel (Thermo Fisher Scientific,
187 Waltham, MA) on a Luminex 200 instrument (Luminex, Austin, TX) in universal assay buffer.
188 Assayed cytokines included: MIP-1α, IL-1β, IL-4, IP-10, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-
189 17A, IFN-γ, GM-CSF, TNF-α, MIP-1β, IFN-α, MCP-1, P-Selectin, IL-1α, sICAM-1, and E-
190 Selectin. Following manufacturer protocols, all samples were run on a plate with 7 standards
191 (diluted 1:4) and a control (universal assay buffer only), with all samples, standards, and
192 controls run in duplicate similar to our methods previously (Pessoa *et al.*, 2019; Kleinstein *et al.*,
193 2020) .
194
195 Quality control (QC) steps were conducted according to manufacturer recommendations by
196 xPONENT 4.2 software (Affymetrix eBioscience, San Diego, USA). Any standards with <70 or
197 >130 % recovery of beads were invalidated. Samples were required to have a bead count of
198 >30 beads recovered (all samples had >100 beads recovered and none were excluded at this
199 step). Following QC, results were reported as average pg/mL for all measured cytokines. The
200 lower limit of quantification (LLOQ) was determined based on the standard curve (after QC) as
201 the average value of the lowest validated standards. Values at or below the LLOQ for each
202 cytokine were reported at the LLOQ. As samples were run in two batches (with similar LLOQs
203 for each batch), LLOQ for the cytokines (in pg/mL) were averaged across both runs: MIP-
204 1α=1.79, IL-1β=5.58, IL-4=23.99, IP-10= 1.17, IL-6=18.89, IL-8=2.48, IL-10=2.01, IL-
205 12p70=11.72, IL-13=5.63, IL-17A=4.63, IFN-γ=11.93, GM-CSF=14.57, TNF-α=9.66, MIP-
206 1β=7.66, IFN-α=1.32, MCP-1=3.73, P-Selectin=1077.15, IL-1α=0.74, sICAM-1=442.80, and E-
207 Selectin=441.00. To compare cytokine levels between study groups, unpaired t-tests were used
208 with a significance threshold of $p<0.05$ and no assumption of consistent standard deviation.
209
210 <u>Minimum Entropy Decomposition analysis of sequence libraries</u>
211
212 Poor quality reads were discarded using flexbar (Dodt *et al.*, 2012). Reads were then treated as
213 single end reads and trimmed for quality and to a length of 160bp using Trimmomatic (Bolger,
214 Lohse and Usadel, 2014). Minimum Entropy Decomposition (MED) was performed as described
215 in the oligotyping pipeline (Eren *et al.*, 2015), generating a list of node representative sequences
216 and their relative abundances. Taxonomic assignment of representative sequences were
217 generated based on aligning 16s rRNA sequences at 98.5% identity against the eHOMD
218 database (Escapa *et al.*, 2018) and further evaluated using the Ribosomal Database Project
219 (RDP) database (Cole *et al.*, 2014). MED nodes were assigned species level taxonomy based
220 on >98.5% identity matches to eHOMD and/or RDP with eHOMD designations used as 1st
221 priority as our intial aim was to identify oral taxa. Node frequency tables now assigned
222 taxonomy were then used in association with sample metadata for Linear Discriminate Effect
223 Size analysis (LEfSe) (Segata *et al.*, 2011).

224 <u>QIIME2 analysis of sequence libraries</u>
225

226 *All Scripts at end of this document. All work was performed on the University of Rhode Island
227 High Performance Computing Bluewaves cluster:

228 https://web.uri.edu/hpc-research-computing/cluster-specifications/

229 *Data Import*

230 Fastq sequence data were imported using the **import-fastq.sh** script. Generated demux.qzv
231 files were viewed using https://view.qiime2.org/ via the "quality viewer" function to determine
232 location for sequence quality trimming used in the following step.

233 *Fastq Trimming, DADA2 Analysis, Run-run Merging*

234 A second sequencing run of the prepared Illumina libraries was performed to ensure maximum
235 sequencing depth without further PCR amplification of starting template. At the end of the "D2-
236 merge.sh" script we then used the **Rarefaction-alpha.sh** script output (Fig. S2) to determine
237 read depth (22,000) to use in downstream commands.

238 *Initial Alpha and Beta Diversity Measurements and Run-run Comparison Testing*

239 Merged libraries were used in the **Alpha-Beta-An.sh** script to determine initial phylogenetics on
240 unfiltered data using the QIIME2 *core-metrics-phylogenetic* function, Alpha and Beta diversity
241 calculations via QIIME2 *alpha-* or *beta-group-significance* functions as well as Beta diversity
242 difference calculation (*beta-group-significance*) between sequencing Run 1 vs. Run 2 (Figs. S3,
243 S4).

244 *Mock Community Standards Quality Control Testing*

245 This section uses the **mocklibQC1.sh** and **mocklibQC2.sh** scripts. Taxonomy files used are
246 described below. The *-bar-plots.qzv file generated in the 1st script was viewed at
247 https://view.qiime2.org/ and the level 6 data from this plot was exported as a .csv file which
248 provides the reads per each taxa. This data was converted to proportion of total reads per each
249 taxa and converted into the Zymo_actual.tsv file. The Zymo_expected.tsv file was generated by
250 editing the Zymo_actual.tsv file with the proportion for each taxa present as provided in the
251 manufacturers reference:
252 https://files.zymoresearch.com/protocols/_d6305_d6306_zymobiomics_microbial_community_d
253 na_standard.pdf

254 These .tsv inputs were then used in the **mocklibQC2.sh** script to output the visualizations seen
255 in Figure S1.

256 *Classifier Setup and Editing*

257 Initial import of the SILVA 132 classifier was performed by running the following command:

258 *wget https://data.qiime2.org/2019.7/common/silva-132-99-nb-classifier.qza*

259 Classifier setup was then performed using the **classifier.sh** script. Upon searching the silva-
260 taxonomy.qzv file generated, we noted that there were no reads that matched *A. baumanii*
261 sequences at all. We used the rep-seqs.merge.qzv file output from the **D2merge.sh** script
262 saved as a FASTA file from within the QIIME2 viewer function and performed a pairwise blast vs
263 the full length *A. baumanii* 16S sequence from NCBI:

264 >A.baumanii 16s

265 AACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGGGGAAGGTAGCTTGCTACCGG
266 ACCTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATC
267 TCGAAAGGGATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCT
268 TGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGG
269 CGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCC

270 AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAACCCTGATCCAG
271 CCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGC
272 TACTTTAGTTAATACCTAGAGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCT
273 GTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAA
274 GCGTGCGTAGGCGGCTTATTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGC
275 ATTCGATACTGGTGAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGA
276 AATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGAC
277 GCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
278 AACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAG
279 TAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGC
280 ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGA
281 CATACTAGAAACTTTCCAGAGATGGATTGGTGCCTTCGGGAATCTAGATACAGGTGCTGCA
282 TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
283 TTTCCTTACTTGCCAGCATTTCGGATGGGAACTTTAAGGATACTGCCAGTGACAAACTGGA
284 GGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCT
285 ACAATGGTCGGTACAAAGGGTTGCTACACAGCGATGTGATGCTAATCTCAAAAAGCCGATC
286 GTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGG
287 ATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGG
288 GAGTTTGTTGCACCAGAAGTAGCTAGCCTAACTGCAAAGAGGGCGGTTACCACGGTGTGG
289 CCGATGACTGGGGTGAAGT

290 One ASV from the rep-seqs.merge.qzv file (e875355f3179838110485d8f5013d4a6) returned a
291 100% match homology to the above *A. baumanii* sequence. This ASV was just annotated as
292 *Acinetobacter* in the SILVA-132-99 taxonomy. We then edited the silva classifier taxonomy file
293 by first exporting it using the following commands:

294 *module load QIIME2/2019.7*

295 *qiime tools export *

296 *--input-path silva-taxonomy.qza *

297 *--output-path silva-taxonomy*

298 This output the file in *.tsv format which was then opened in a text editor and searched for the
299 ASV node identity and then modified as indicated here:

300 **before**

301 e875355f3179838110485d8f5013d4a6
302 D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D
303 _4__Moraxellaceae;D_5__Acinetobacter   0.9999662516375396

304 **after**

305 e875355f3179838110485d8f5013d4a6
306 D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D
307 _4__Moraxellaceae;D_5__Acinetobacter;D_6__Acinetobacter baumanii
308 0.9999662516375396

309 The edited file was then saved as taxonomy-1.tsv and imported via the following command:

310 *qiime tools import *

311 *--input-path /data/mramseylab/classifiers/silva-taxonomy/taxonomy-1.tsv *

312 *--type 'FeatureData[Taxonomy]' *

313 *--input-format TSVTaxonomyFormat *

314 *--output-path silva-mod-taxonomy.qza*

315 *qiime metadata tabulate *

316 *--m-input-file silva-mod-taxonomy.qza *

317 *--o-visualization silva-mod-taxonomy.qzv*

318 The silva-mod-taxonomy.qza file was used for the rest of our analyses.

319 *Data Filtering*

320 Data presented throughout the manuscript is generally described as "Minimally Filtered" or
321 "Strict Filtered". We 1st began with all ASV assigned data from the above scripts and used
322 metadata based filtering to separate out all data from control samples which included no-
323 template and PCR only control indexes. This was done using the **control-filter.sh** script. Data
324 output from the script was exported so it could be viewed and then used to subtract taxa from
325 human-derived datasets in downstream filtering steps. Next we applied the **meta-filter.sh** script
326 to our data to extract only human derived samples for further analysis and filtering. After this we
327 applied the **minimal-filter.sh** script. Outputs from this script were used in all "Minimally Filtered"
328 described data in the manuscript. Minimal filtering included removal of instances of taxa that
329 appeared in only 2 samples or less, features (ASV sequences) that appeared less than 10 times
330 across all samples and any taxa with less than 20 reads per sample.

331 Next we took our output files from the above **control-filter.sh** script and used them as part of
332 the input for the **auto-filter.sh** script to exclude these taxa from the remaining minimally filtered
333 data. While this removed many spurious taxa from our samples we observed high abundance of
334 known aquatic microbial contaminant sequences not typically human associated (ex:
335 *Sphingomonas, Ralstonia*). These and other taxa were manually excluded using the **strict-**
336 **filter.sh** script and further removal of highly abundant *Pseudomonas* sequences were also
337 removed via the **nopa-filter.sh** script. Data filtered to this extent are referred to as "Strictly"
338 filtered in the manuscript.

339 *Re-analysis of Filtered Data*

340 A reanalysis of filtered data was performed 1st for Alpha and Beta diversity measurements via
341 the **alpha-beta2.sh** script (Fig. 1). Further description and statistical analysis of Beta diversity
342 differences were performed in R using the *vegan* package via the metaMDS and Adonis
343 functions primarily as demonstrated in the **MMR20_ellipses_NMDS.R** script below.

344 *LEfSe Analysis Comparison*

345 Initial LEfSe analysis was performed using ASV output abundance data directly from the initial
346 MED pipeline (Fig. 3). Further analysis on QIIME2 / DADA2 assigned strictly filtered data (Fig.
347 S5) was performed first using the **lefse-noTax.sh** script to export and format data for analysis
348 on the LEfSe Galaxy server (https://huttenhower.sph.harvard.edu/galaxy/). This script only
349 outputs ASV node information without taxonomic assignment. Manual taxonomic assignment
350 was performed comparing ASV node names to the rep-seqs-merge.qza file from the

351   **D2merge.sh** script. LEfSe analysis was performed using default settings, *.svg output files were
352   further edited in InkScape software for clarity.

353

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399

400   **Supplemental Data (Data S1.)** Related to Transparent Methods

401   Scripts used for analyses:

402

403   **import-fastq.sh**

404   ```
module load QIIME2/2019.7
```

405

406    ```
for d in /data/mramseylab/raw_reads/2018_Serum/Run* ;
```

407   ```
do
```

408    ```
SUFX=${d#*Run}
```

409

410   ```
qiime tools import \
```

411    ```
  --type 'SampleData[PairedEndSequencesWithQuality]' \
```

412   ```
  --input-path /data/mramseylab/raw_reads/2018_Serum/Run$SUFX \
```

413    ```
  --input-format CasavaOneEightSingleLanePerSampleDirFmt \
```

414   ```
  --output-path /data/mramseylab/raw_reads/2018_Serum/Run$SUFX/demux-
```
415    ```
$SUFX.qza
```

416

417   ```
qiime demux summarize \
```

418    ```
  --i-data /data/mramseylab/raw_reads/2018_Serum/Run$SUFX/demux-
```
419   ```
$SUFX.qza \
```

420    ```
  --o-visualization /data/mramseylab/visualizations/demux-$SUFX.qzv
```

421

422   ```
done
```

423

```
424    D2merge.sh

425

426    module load QIIME2/2019.7

427

428    for d in /data/mramseylab/raw_reads/2018_Serum/Run* ;

429    do

430    SUFX=${d#*Run}

431

432    qiime dada2 denoise-single \

433      --i-demultiplexed-seqs
434    /data/mramseylab/raw_reads/2018_Serum/Run$SUFX/demux-$SUFX.qza \

435      --p-trim-left 13 \

436      --p-trunc-len 250 \

437      --o-table /data/mramseylab/raw_reads/2018_Serum/Run$SUFX/denoise-
438    table-$SUFX.qza \

439      --o-representative-sequences
440    /data/mramseylab/raw_reads/2018_Serum/Run$SUFX/rep-seqs-$SUFX.qza \

441      --o-denoising-stats
442    /data/mramseylab/raw_reads/2018_Serum/Run$SUFX/denoising-stats-
443    $SUFX.qza

444

445    done

446    #Have to MERGE Run1 and Run2 to get pairwise comparison

447    #Must make directory before running below else it will error out.

448

449    mkdir /data/mramseylab/raw_reads/2018_Serum/Merge_runs/

450

451    qiime feature-table merge \

452      --i-tables /data/mramseylab/raw_reads/2018_Serum/Run1/denoise-table-
453    1.qza \

454      --i-tables /data/mramseylab/raw_reads/2018_Serum/Run2/denoise-table-
455    2.qza \
```

```
456    --o-merged-table
457  /data/mramseylab/raw_reads/2018_Serum/Merge_runs/denoise-table-
458  merge.qza

459

460  qiime feature-table merge-seqs \
461    --i-data /data/mramseylab/raw_reads/2018_Serum/Run1/rep-seqs-1.qza \
462    --i-data /data/mramseylab/raw_reads/2018_Serum/Run2/rep-seqs-2.qza \
463    --o-merged-data
464  /data/mramseylab/raw_reads/2018_Serum/Merge_runs/rep-seqs-merge.qza

465

466  qiime feature-table summarize \
467    --i-table /data/mramseylab/raw_reads/2018_Serum/Merge_runs/denoise-
468  table-merge.qza \
469    --o-visualization /data/mramseylab/visualizations/denoise-table-
470  merge.qzv \
471    --m-sample-metadata-file /data/mramseylab/metadata/Serum5.tsv

472

473  qiime feature-table tabulate-seqs \
474    --i-data /data/mramseylab/raw_reads/2018_Serum/Merge_runs/rep-seqs-
475  merge.qza \
476    --o-visualization /data/mramseylab/visualizations/rep-seqs-merge.qzv

477

478  qiime phylogeny align-to-tree-mafft-fasttree \
479    --i-sequences /data/mramseylab/raw_reads/2018_Serum/Merge_runs/rep-
480  seqs-merge.qza \
481    --o-alignment
482  /data/mramseylab/raw_reads/2018_Serum/Merge_runs/aligned-rep-seqs-
483  merge.qza \
484    --o-masked-alignment
485  /data/mramseylab/raw_reads/2018_Serum/Merge_runs/masked-aligned-rep-
486  seqs-merge.qza \
487    --o-tree /data/mramseylab/raw_reads/2018_Serum/Merge_runs/unrooted-
488  tree-merge.qza \
489    --o-rooted-tree
490  /data/mramseylab/raw_reads/2018_Serum/Merge_runs/rooted-tree-merge.qza
```

Rarefaction-alpha.sh

```
module load QIIME2/2019.7

rawdir=/data/mramseylab/raw_reads/2018_Serum/Merge_runs/
procdir=/data/mramseylab/proc_reads/
metadir=/data/mramseylab/metadata/
visdir=/data/mramseylab/visualizations/

qiime diversity alpha-rarefaction \
  --i-table $rawdir\denoise-table-merge.qza \
  --i-phylogeny $rawdir\rooted-tree-merge.qza \
  --p-max-depth 25000 \
  --m-metadata-file $metadir\Serum4.tsv \
  --o-visualization $visdir\alpha-rarefaction.qzv
```

Alpha-Beta-An.sh

```
module load QIIME2/2019.7

rawdir=/data/mramseylab/raw_reads/2018_Serum/Merge_runs/
procdir=/data/mramseylab/proc_reads/
metadir=/data/mramseylab/metadata/
cmr="core-metrics-results"

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny $rawdir\rooted-tree-merge.qza \
  --i-table  $rawdir\denoise-table-merge.qza \
  --p-sampling-depth 22000 \
  --m-metadata-file $metadir\Serum5.tsv \
  --output-dir $procdir$cmr\-initial

qiime diversity alpha-group-significance \
  --i-alpha-diversity $procdir$cmr\-initial/faith_pd_vector.qza \
  --m-metadata-file $metadir\Serum5.tsv \
  --o-visualization $procdir$cmr\-initial/faith-pd-group-
significance.qzv

qiime diversity alpha-group-significance \
  --i-alpha-diversity $procdir$cmr\-initial/evenness_vector.qza \
  --m-metadata-file $metadir\Serum5.tsv \
  --o-visualization $procdir$cmr\-initial/evenness-group-
significance.qzv

array=( unweighted_unifrac_distance_matrix
weighted_unifrac_distance_matrix bray_curtis_distance_matrix )
```

```
541  for i in "${array[@]}"

542  do

543

544  qiime diversity beta-group-significance \

545    --i-distance-matrix $procdir$cmr\-initial/$i.qza \

546    --m-metadata-file  $metadir\Serum5.tsv \

547    --m-metadata-column Condition \

548    --o-visualization $procdir$cmr\-initial/$i.qzv \

549    --p-pairwise

550

551  done

552

553  #Generates data for run1 vs run2 variability to measure batch effect

554  qiime diversity beta-group-significance \

555    --i-distance-matrix $procdir$cmr\-
556  initial/unweighted_unifrac_distance_matrix.qza \

557    --m-metadata-file $metadir\Serum4.tsv \

558    --m-metadata-column Run \

559    --o-visualization $procdir$cmr\-initial/unweighted-unifrac-Run-
560  significance.qzv \

561    --p-pairwise

562
```

563 **Classifier.sh**

564 ```
module load QIIME2/2019.7
```

565

566 ```
rawdir=/data/mramseylab/raw_reads/2018_Serum/Merge_runs/
```

**567** ```
clsdir=/data/mramseylab/classifiers/
```

568

569 ```
#for the silva release 132 99 .fna file
```

**570**

**571** ```
qiime feature-classifier classify-sklearn \
```

572 ```
  --i-classifier $clsdir\silva-132-99-nb-classifier.qza \
```

**573** ```
  --i-reads $rawdir\rep-seqs-merge.qza \
```

574 ```
  --o-classification $clsdir\silva-taxonomy.qza
```

**575**

**576** ```
#output the taxonomy table to check for A. baumanii strains in it
```
577 ```
afterwards.
```

**578**

**579** ```
qiime metadata tabulate \
```

580 ```
  --m-input-file $clsdir\silva-taxonomy.qza \
```

**581** ```
  --o-visualization $clsdir\silva-taxonomy.qzv
```

582

583 **Control-filter.sh**

584 `module load QIIME2/2019.7`

585

586 `tablein=/data/mramseylab/raw_reads/2018_Serum/Merge_runs/denoise-`
587 `table-merge.qza`

588 `clsdir=/data/mramseylab/classifiers/`

589 `metadir=/data/mramseylab/metadata/`

590 `visdir=/data/mramseylab/visualizations/`

591 `filtdir=/data/mramseylab/proc_reads/`

592 `# filter status of input files, "ctrl-filter" is just for taxa`
593 `belonging to no template controls`

594 `fil=ctrl-filter`

595

596

597 `#must make the directory you are filtering to 1st or else it will`
598 `error`

599 `mkdir $filtdir$fil`

600

601

602 `qiime feature-table filter-samples \`

603 `  --i-table $tablein \`

604 `  --m-metadata-file $metadir\Serum5.tsv \`

605 `  --p-where "[Source]='Control'" \`

606 `  --o-filtered-table $filtdir$fil/$fil-table.qza`

607

608 `qiime taxa collapse \`

609 `  --i-table $filtdir$fil/$fil-table.qza \`

610 `  --i-taxonomy $clsdir\silva-mod-taxonomy.qza \`

611 `  --p-level 6 \`

612 `  --o-collapsed-table $filtdir$fil/$fil-collapse-table.qza`

```
613
614   qiime feature-table relative-frequency \
615     --i-table $filtdir$fil/$fil-collapse-table.qza \
616     --o-relative-frequency-table $filtdir$fil/$fil-relative-collapse-
617   table.qza
618
619   qiime tools export \
620     --input-path $filtdir$fil/$fil-relative-collapse-table.qza \
621     --output-path $filtdir$fil/
622
623   biom convert \
624   -i $filtdir$fil/feature-table.biom \
625   -o $filtdir$fil/$fil-relative-collapse-table.txt \
626   --header-key "taxonomy" \
627   --to-tsv
628
629   #Use above taxa table to filter out based on taxa present in controls
630
631   qiime feature-table filter-features \
632     --i-table $tablein \
633     --m-metadata-file $filtdir$fil/$fil-collapse-table.qza \
634     --o-filtered-table $filtdir$fil/$fil-excluded-table.qza \
635     --p-exclude-ids
636
637   #Use excluded table to generate barplot for checking
638
639   qiime taxa barplot \
640     --i-table $filtdir$fil/$fil-excluded-table.qza \
641     --i-taxonomy $clsdir\silva-mod-taxonomy.qza \
```

```
642      --m-metadata-file $metadir\Serum5.tsv \
643      --o-visualization $filtdir$fil/$fil-excluded-table.qzv
644
```

Meta-filter.sh

```
module load QIIME2/2019.7


tablein=/data/mramseylab/raw_reads/2018_Serum/Merge_runs/denoise-
table-merge.qza

clsdir=/data/mramseylab/classifiers/

metadir=/data/mramseylab/metadata/

visdir=/data/mramseylab/visualizations/

filtdir=/data/mramseylab/proc_reads/

# filter status of input files, "initial" is the 1st pass no filter at
all

# "initial-human" is the initial data but for only human samples, no
mock or control samples

fil=initial-human


#note must make directores needed BEFORE running the below command

mkdir $filtdir$fil


qiime feature-table filter-samples \
  --i-table $tablein \
  --m-metadata-file $metadir\Serum5.tsv \
  --p-where "[Source]='Human'" \
  --o-filtered-table $filtdir$fil/$fil-table.qza
```

669 **Auto-filter.sh**

670 `module load QIIME2/2019.7`

671

672 `tablein=/data/mramseylab/proc_reads/minF-hum/table3.qza`

673 `clsdir=/data/mramseylab/classifiers/`

674 `metadir=/data/mramseylab/metadata/`

675 `visdir=/data/mramseylab/visualizations/`

676 `filtdir=/data/mramseylab/proc_reads/`

677

678 `# filter status of input files, "ctrl-filter" is just for taxa`
679 `belonging to no template controls`

680 `fil=AF-hum`

681 `#must make the directory you are filtering to 1st or else it will`
682 `error`

683 `mkdir $filtdir$fil`

684

685 `#Filtering list from mock samples was generated previously with the`
686 `control-filter.sh script`

687

688 `qiime feature-table filter-features \`

689 `  --i-table $tablein \`

690 `  --m-metadata-file /data/mramseylab/proc_reads/ctrl-filter/ctrl-`
691 `filter-collapse-table.qza \`

692 `  --o-filtered-table $filtdir$fil/$fil-excluded-table.qza \`

693 `  --p-exclude-ids`

694

695 `#Use excluded table to generate barplot for checking`

696 `qiime taxa barplot \`

697 `  --i-table $filtdir$fil/$fil-excluded-table.qza \`

698 `  --i-taxonomy $clsdir\silva-mod-taxonomy2.qza \`

699 `  --m-metadata-file $metadir\Serum5.tsv \`

```
700     --o-visualization $filtdir$fil/$fil-excluded-table.qzv

701

702     #Generate viewable feature table to look for ASVs of interest /
703     filtering stats

704     qiime feature-table summarize \

705       --i-table $filtdir$fil/$fil-excluded-table.qza \

706       --o-visualization $filtdir$fil/$fil-excluded-table2.qzv \

707       --m-sample-metadata-file $metadir\Serum5.tsv

708
```

709 **Strict-filter.sh**

710 `module load QIIME2/2019.7`

711

712 `tablein=/data/mramseylab/proc_reads/AF-hum/AF-hum-excluded-table`

713 `clsdir=/data/mramseylab/classifiers/`

714 `metadir=/data/mramseylab/metadata/`

715 `visdir=/data/mramseylab/visualizations/`

716 `filtdir=/data/mramseylab/proc_reads/`

717

718 `# filter status of input files, "ctrl-filter" is just for taxa`
719 `belonging to no template controls`

720 `fil=SF-hum`

721 `#must make the directory you are filtering to 1st or else it will`
722 `error`

723 `mkdir $filtdir$fil`

724

725 `#Filtering list from mock samples was generated previously with the`
726 `auto-filter.sh script`

727

728 `#Tidying up name here so I can repeatedly use the same input below`
729 `through the loop`

730 `cp $tablein.qza /$filtdir$fil/$fil-table.qza`

731 `#Array here contains all taxa I wish to remove`

732 `array=( Chloroplast Mitochondria Ralstonia Chryseobacterium`
733 `Sphingomonas Enhydrobacter Bradyrhizobium Sphingomonadales Rhizobiales`
734 `Rhodobacterales Sphingobacteriales Halomonadaceae Deinococcales )`

735

736 `for i in "${array[@]}"`

737 `do`

738

739 `qiime taxa filter-table \`

```
740     --i-table /$filtdir$fil/$fil-table.qza \

741     --i-taxonomy $clsdir\silva-mod-taxonomy2.qza \

742     --p-mode contains \

743     --p-exclude "$i" \

744     --o-filtered-table /$filtdir$fil/$fil-table.qza

745

746  done

747  #Use excluded table to generate barplot for checking

748  qiime taxa barplot \

749     --i-table /$filtdir$fil/$fil-table.qza \

750     --i-taxonomy $clsdir\silva-mod-taxonomy2.qza \

751     --m-metadata-file $metadir\Serum5.tsv \

752     --o-visualization /$filtdir$fil/$fil-table-barplot.qzv

753

754  #Generate viewable feature table to look for ASVs of interest /
755  filtering stats

756  qiime feature-table summarize \

757     --i-table /$filtdir$fil/$fil-table.qza \

758     --o-visualization /$filtdir$fil/$fil-table.qzv \

759     --m-sample-metadata-file $metadir\Serum5.tsv

760
```

761 **Nopa-filter.sh**

762 ```
module load QIIME2/2019.7
```

763

764 ```
tablein=/data/mramseylab/proc_reads/SF-hum/SF-hum-table.qza
```

765 ```
clsdir=/data/mramseylab/classifiers/
```

766 ```
metadir=/data/mramseylab/metadata/
```

767 ```
visdir=/data/mramseylab/visualizations/
```

768 ```
filtdir=/data/mramseylab/proc_reads/
```

769

770 ```
filter status of input files, "ctrl-filter" is just for taxa
771 belonging to no template controls
```

772 ```
fil=NP-hum
```

773 ```
#must make the directory you are filtering to 1st or else it will
774 error
```

775 ```
mkdir $filtdir$fil
```

776

777 ```
#Filtering list from mock samples was generated previously with the
778 strict-filter.sh script
```

779

780 ```
qiime taxa filter-table \
```

781 ```
  --i-table $tablein \
```

782 ```
  --i-taxonomy $clsdir\silva-mod-taxonomy2.qza \
```

783 ```
  --p-mode contains \
```

784 ```
  --p-exclude "Pseudomonas" \
```

785 ```
  --o-filtered-table $filtdir$fil/$fil-table.qza
```

786

787 ```
#Use excluded table to generate barplot for checking
```

788 ```
qiime taxa barplot \
```

789 ```
  --i-table $filtdir$fil/$fil-table.qza \
```

790 ```
  --i-taxonomy $clsdir\silva-mod-taxonomy2.qza \
```

```
791    --m-metadata-file $metadir\Serum5.tsv \

792    --o-visualization $filtdir$fil/$fil-table-barplot.qzv

793

794    #Generate viewable feature table to look for ASVs of interest /
795    filtering stats

796    qiime feature-table summarize \

797    --i-table $filtdir$fil/$fil-table.qza \

798    --o-visualization $filtdir$fil/$fil-table.qzv \

799    --m-sample-metadata-file $metadir\Serum5.tsv

800
```

801 **Alpha-beta2.sh**

802 ```
module load QIIME2/2019.7
```

803

804 ```
rawdir=/data/mramseylab/raw_reads/2018_Serum/Merge_runs/
```

**805** ```
procdir=/data/mramseylab/proc_reads/
```

806 ```
metadir=/data/mramseylab/metadata/
```

**807** ```
visdir=/data/mramseylab/visualizations/
```

808 ```
cmr="core-metrics-results"
```

**809**
**810** ```
# filter status of input files, "initial" is the 1st pass no filter at
all
```

811 ```
fil=-initial
```

**812**

**813**
**814** ```
#note change name of input tables for the 1st command below. Some
input tables did not have standardized filename conventions
```

815 ```
#changed to AF-hum-table.qza and table3.qza to minF-hum-table.qza
```

**816**

**817** ```
array=( minF AF SF NP )
```

818

819 ```
for i in "${array[@]}"
```

**820** ```
do
```

821

822 ```
qiime diversity core-metrics-phylogenetic \
```

**823** ```
  --i-phylogeny $rawdir\rooted-tree-merge.qza \
```

824 ```
  --i-table $procdir$i\-hum/$i\-hum-table.qza \
```

**825** ```
  --p-sampling-depth 5000 \
```

826 ```
  --m-metadata-file $metadir\Serum5.tsv \
```

**827** ```
  --output-dir $procdir$i\-hum/$cmr
```

828

829 ```
qiime diversity alpha-group-significance \
```

**830** ```
  --i-alpha-diversity $procdir$i\-hum/$cmr/faith_pd_vector.qza \
```

```
831    --m-metadata-file $metadir\Serum5.tsv \

832    --o-visualization $procdir$i\-hum/$cmr/faith-pd-group-
833    significance.qzv

834

835    qiime diversity alpha-group-significance \

836      --i-alpha-diversity $procdir$i\-hum/$cmr/evenness_vector.qza \

837      --m-metadata-file $metadir\Serum5.tsv \

838      --o-visualization $procdir$i\-hum/$cmr/evenness-group-
839    significance.qzv

840

841    done

842

843    #dual array taking the directories above and then running the next
844    command on the 3 filenames in array2 for each directory prefix in
845    array 1

846    #note different array command syntax from above vs below

847

848    array1=( minF AF SF NP )

849    array2=( unweighted_unifrac_distance_matrix
850    weighted_unifrac_distance_matrix bray_curtis_distance_matrix )

851    for indirs in ${array1[@]}

852    do

853        for infils in ${array2[@]}

854        do

855

856    qiime diversity beta-group-significance \

857      --i-distance-matrix $procdir$indirs\-hum/$cmr/$infils\.qza \

858      --m-metadata-file $metadir\Serum5.tsv \

859      --m-metadata-column Condition \

860      --o-visualization $procdir$indirs\-hum/$cmr/$infils\.qzv \

861      --p-pairwise
```

862   done

863   done

864

865 **MMR20_ellipses_NMDS.R**

```r
866  setwd("E:/2019_Diabetic_Serum/Figure Materials/R-Plots/")

867  library(vegan)

868  library(ggplot2)

869  library(dplyr)

870  set.seed(30)

871

872  metadata<-read.csv("Serum5.tsv", header=TRUE, sep="\t",
873  stringsAsFactors = F)

874  featuredf<-read.csv("NP-hum-features.tsv", header=TRUE, sep="\t",
875  stringsAsFactors = F)

876

877  #making a subset of human only metadata and a few other criteria.

878  metadata <- metadata %>% filter(Source == 'Human') %>%
879  select("sample.id","Condition","Run","Source","ABC")

880

881  #Fixing 1st row to make it row names

882  featuredf <- data.frame(featuredf[,-1], row.names = featuredf[,1])

883

884  #transposing trial1 table to match Evelyns data format

885  featuredf <- as.data.frame(t(featuredf))

886

887  #Brings tables into agreement on matching sample.id values

888  featuredf = featuredf %>% mutate(sample.id = rownames(featuredf))

889

890  #Brings tables into agreement on matching sample.id values

891  table_all = left_join(featuredf, metadata)

892

893  #This gets metadata to agree with row numbers of the features table
894  after splitting out from table_all
```

```r
895   metadata <-
896   select(table_all,"sample.id","Condition","Run","Source","ABC")

897

898   #filter the "sample.id" column off the very end of the dataframe and
899   turn it into rownames

900   #NOTE THIS VALUE MUST BE CHANGED FOR EACH INPUT

901   featuredf <- data.frame(featuredf[,-1101], row.names =
902   featuredf[,1101])

903

904   # normalize data by sum of ASVs in each sample (this was from ZP's
905   code)

906   featuredf <- sweep(featuredf,2,colSums(featuredf),`/`)

907

908   ##Above this was all data input / manipulation, Below this is data
909   analysis and plotting##

910

911   ### The function metaMDS is used to calculate the dissimilarity matrix
912   using the bray curtis distance metrics and at the same

913   #time generates the values from the dissimilarity matrix for  an
914   ordination plot.

915   MDS <-metaMDS(featuredf ,distance = "bray", k = 3, trymax = 500)

916

917   ## Next, extract the x and y coordinates from the MDS plot into a new
918   data frame and add the metadata factors to the coordinates the data
919   should be plotted based on.

920   #MMR- added ABC=as.factor and changed [,3] to [,5] to agree with my
921   own metadata file.

922   NMDS_t1=data.frame(NMDS1=MDS$point[,1],NMDS2=MDS$point[,2],

923                    Condition=table_all$Condition,ABC=table_all$ABC)

924

925   ## set theme for following plots

926   theme_set(theme_bw())

927
```

```r
928   ## Generate the ordination based on the solution from above and
929   selected grouping factor (Condition)

930   plot.new()

931   ord<-ordiellipse(MDS, table_all$Condition,

932                        display = "sites", kind ="sd", conf = 0.95, label
933   = T)

934   dev.off()

935

936   ## Data frame df_ell_t1 contains values to show ellipses. It is
937   calculated with function veganCovEllipse which is hidden in vegan
938   package.

939   #This function is applied to each level of NMDS (group) and it uses
940   also function cov.wt to calculate covariance matrix.

941   veganCovEllipse<-function (cov, center = c(0, 0), scale = 1, npoints =
942   100)

943   {

944     theta <- (0:npoints) * 2 * pi/npoints

945     Circle <- cbind(cos(theta), sin(theta))

946     t(center + scale * t(Circle %*% chol(cov)))

947   }

948

949   ##Generate ellipse points based on 95% confidence (SD) intervals

950   #Reference : http://stackoverflow.com/questions/13794419/plotting-
951   ordiellipse-function-from-vegan-package-onto-nmds-plot-created-in-
952   ggplot

953   #Data frame df_ell contains values to show ellipses. It is calculated
954   with function veganCovEllipse which is hidden in vegan package. This
955   function is applied to each level of NMDS (group) and it uses also
956   function cov.wt to calculate covariance matrix.

957   df_ell_t1 <- data.frame()

958   for(g in levels(NMDS_t1$Condition)){

959     if(g!="" && (g %in% names(ord))){

960       df_ell_t1 <- rbind(df_ell_t1,
961   cbind(as.data.frame(with(NMDS_t1[NMDS_t1$Condition==g,],
```

```r
962
963    veganCovEllipse(ord[[g]]$cov,ord[[g]]$center,ord[[g]]$scale))),Conditi
964    on=g))}}
965
966    ## Calculate p-value:
967    adon_t1<-adonis2(featuredf ~Condition, data=metadata,
968    by=NULL,method="bray", k=3)
969
970    NMDSplot_t1<-ggplot(data=NMDS_t1,aes(NMDS1,NMDS2,col=Condition))+
971        #update from Evelyn to add metadata text to plot if wanted
972      #This is useful to identify outlier points if using sample ID text
973    here, Remove for final plot
974    ###
975    geom_text(aes(NMDS1,NMDS2,label=table_all$sample.id),size=2,vjust=0)+
976      # add the p-value in the bottom right corner
977      annotate("text",x=min(NMDS_t1$NMDS1),y=min(NMDS_t1$NMDS2-0.5),
978              label=paste("p= ", adon_t1$`Pr(>F)`[1]),size=3)+
979      # draw the ellipses and define color based on the grouping factor
980      geom_path(data=df_ell_t1, aes(x=NMDS1, y=NMDS2, linetype=Condition),
981    size=1)+
982      #scale_linetype_manual(values=c("4-Con"="dotted","3-S4"="solid","2-
983    RI-old"="longdash"))+
984      #scale_colour_manual(values=c("4-Con"="red", "3-S4"="darkgreen", "2-
985    RI-old"="purple"))+
986      # add the points per sample and define shape based on TankLocation
987      geom_point(aes(shape=ABC), size=2) +
988      # Reorder the legend
989      guides(color = guide_legend(order=1),lty= guide_legend(order=1),
990            shape = guide_legend(order=2), legend.position = "bottom")
991
992    # Adding other Aethestics
```

```
993   NMDS_by_Trial1 <- NMDSplot_t1 + theme(axis.text.y =
994   element_text(size="12", color="black"), axis.title.y =
995   element_text(face="bold",size="12", color="black"))+

996     theme(axis.text.x = element_text(size="12", color="black"),
997   axis.title.x.bottom = element_text(face="bold",size="12",
998   color="black"))+

999     theme(axis.text.x.top= element_text(face="bold",size="12",
1000  color="black")) +

1001    #change name of the legend

1002    theme(legend.title=element_blank())+

1003    theme(legend.position = "right", legend.title =
1004  element_text(colour="black", size=16, face="bold"))

1005

1006

1007  print(NMDS_by_Trial1)

1008  print(NMDSplot_t1)

1009
```

Lefse-noTax.sh

```
module load QIIME2/2019.7


clsdir=/data/mramseylab/classifiers/
metadir=/data/mramseylab/metadata/
visdir=/data/mramseylab/visualizations/
filtdir=/data/mramseylab/proc_reads/
rawdir=/data/mramseylab/raw_reads/2018_Serum/Merge_runs/
procdir=/data/mramseylab/proc_reads/


array=( minF-hum AF-hum SF-hum NP-hum )


for i in "${array[@]}"
do


qiime feature-table relative-frequency \
 --i-table $filtdir$i/$i\-table.qza \
 --o-relative-frequency-table $filtdir$i/$i\-table.notax.qza


qiime tools export \
 --input-path $filtdir$i/$i\-table.notax.qza \
 --output-path $filtdir$i/


#note, must use single hashes for -i / -o unlike other qiime commands.
biom convert \
 -i $filtdir$i/feature-table.biom \
 -o $filtdir$i/$i\-notax.table.txt \
 --header-key "taxonomy" \
 --to-tsv
```

```
1039
1040    done
1041
1042
```