



## Obligate anaerobes are abundant in human necrotizing soft tissue infection samples – a metagenomics analysis

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Necrotizing soft tissue infections (NSTIs) are associated with high morbidity and mortality and are increasing in incidence. Proper identification of the microbial causes of NSTIs is a crucial step in diagnosis and treatment, but the majority of data collected are culture based, which is biased against fastidious organisms, including obligate anaerobes. The goal of this study was to address this gap in knowledge by characterizing NSTI microbial communities through molecular diagnostics. We performed 16S rRNA sequencing on human NSTI samples and identified five genera most commonly found in NSTIs (*Prevotella*, *Bacteroides*, *Peptoniphilus*, *Porphyromonas*, and *Enterococcus*). We found that a >70% contribution of obligate anaerobes to the bacterial population distribution was associated with NSTI mortality, and that NSTI samples, from both survivors and non-survivors, had an increased relative abundance of gram negative bacteria compared to those of abscess patients. Based on our data, we conclude that obligate anaerobes are abundant in NSTIs and a high relative abundance of anaerobes is associated with a worse outcome. We recommend increasing anaerobe antibiotic coverage during the treatment of NSTIs even when anaerobes are not found by traditional clinical microbiology methods, and especially when there is a clinical suspicion for anaerobe involvement.

Key words: Obligate anaerobes; 16S rRNA sequencing; Necrotizing soft tissue infection (NSTI); Molecular diagnostics; Skin and soft tissue infection (SSTI).

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Skin and soft tissue infections (SSTIs) comprise a spectrum of infections. On the mild end of the spectrum are abscesses, which are infections contained within a wall of host matrix that require minimal medical attention. Acute bacterial skin and skin structure infections (ABSSSIs, previously known as complicated SSTIs) are a subset of SSTIs where deeper tissue layers are involved or significant surgical intervention is needed (1). A further subset of ABSSSIs is a group of infections known as necrotizing soft tissue infections (NSTIs). NSTIs are rapidly progressing infections associated with high morbidity and approximately 10–30% mortality (2), despite aggressive surgical debridement of non-viable tissue. The current understanding of the pathophysiology,

diagnosis, and management of NSTIs has been extensively reviewed (3).

According to culture-based data, the bacterial etiology of both abscesses and NSTIs mostly include gram positive cocci, such as *Staphylococcal* and *Streptococcal* species (4–6), and are predominantly polymicrobial (7–9). However, if the bacterial etiologies of these two types of infections are similar, it is unclear why some patients are able to contain their infection within an abscess, while others develop NSTIs, or worse, succumb to their infections. One theory is that the host determines the trajectory of the infection process. Patients with co-morbidities, such as diabetes and obesity, or those who are immunosuppressed, are more susceptible to NSTIs than those without significant co-morbidities or immunosuppression (10–12).

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However, NSTIs also occur in seemingly healthy patients (11, 12). Another theory is that the pathogenesis of the etiologic agent determines infection outcome. For example, some bacterial strains, such as USA300, a methicillin-resistant *Staphylococcus aureus* (MRSA) strain, produce more virulence factors compared to other *S. aureus* strains (13). However, these more virulent strains are also known to cause abscesses, and not all strains isolated from NSTIs possess candidate virulence determinants (14, 15). A third theory is that there are unidentified or underappreciated bacterial species that influence the course of infection and result in an NSTI rather than an abscess. If true, these bacterial species are likely fastidious and either difficult or impossible to grow in the laboratory.

In this study, we explore this third possibility, because unlike many other infection processes, the bacterial etiology of NSTIs is not well understood. The vast majority of data that have been obtained are from identification by culturing methods, which are biased against fastidious organisms and blind to uncultivable bacteria. Thus, our knowledge about the cause of NSTIs is incomplete at best and largely misrepresented at worst. The purpose of this study was to use Next Generation Sequencing to characterize the underlying bacterial etiology of the infections at either end of the SSTI spectrum: abscesses and NSTIs, as this understanding is a crucial first step to future studies on determining risk factors for developing NSTIs from abscesses, as well as early diagnosis and proper treatment of NSTIs.

## METHODS

### Patients

Study participants were recruited through two separate studies approved by the Texas Tech University Health Sciences Center (TTUHSC) Institutional Review Board (IRB). NSTI patients (IRB# L14-002) admitted to an American Burn Association verified center between December 2013 and September 2017 were included. Clinical data were obtained for 28 patients who consented to participate in the study and molecular data were obtained for 25 of these NSTI patients from whom sufficient samples for sequencing were obtained. We recruited 20 patients with confirmed abscesses (IRB# L14-043) at the same tertiary teaching institution from February 2015 to May 2017. Clinical data were obtained for all 20 recruited abscess patients, while molecular data were obtained for only 18 of these patients whose abscess samples provided enough DNA for sequencing. For both of our studies, inclusion criteria were any patient with a NSTI or abscess diagnosis at at least 18 years of age. We excluded patients not willing to provide samples for sequencing. Agreeing or refusing to take part in this study did not alter their clinical treatment. The study was monitored by an

independent Clinical Research Institute (CRI) officer for proper compliance with IRB protocol and data collection.

### Sample collection and storage

#### NSTIs

Three samples of debrided wound tissue were collected in the operating room (OR) and immediately submerged in RNALater<sup>®</sup> (ThermoFisher, Waltham, MA). RNALater<sup>®</sup> was allowed to perfuse the sample at 4 °C for 24–48 h before long-term storage in a –80 °C freezer. Abscesses: Three samples of abscess fluid or swabs were placed RNALater<sup>®</sup> and immediately frozen at –80 °C since no perfusion was necessary.

### Clinical microbiology

After debridement, NSTI samples were sent to the Clinical Microbiology Laboratory at the University Medical Center, Lubbock, Texas, as per standard of care. The samples were both gram stained and initially grown on applicable growth medium under aerobic and anaerobic conditions, per Clinical Laboratory Standards Institute (CLSI) guidelines. Bacteria were identified by mass spectrometry using the Bruker MALDI Biotyper CA System, a Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) identification system.

### DNA extraction

#### NSTI samples

DNA was extracted via ethanol precipitation, following an established protocol (16). In short, samples were lysed by bead-beating, chloroform was added, samples were then shaken vigorously and centrifuged. After removing the aqueous layer, we added Tris-EDTA buffer and shook vigorously. Samples were mixed by rotation and centrifuged. The aqueous phase was transferred to a new tube and was mixed with ethanol, sodium acetate, and linear acrylamide and allowed to precipitate at –80 °C for at least 4 h. Precipitated DNA was pelleted and washed before being dried and resuspended in TE buffer, and finally stored at –80 °C.

#### Abscess samples

Genomic DNA was extracted from samples using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) following manufacturer's protocol at RTLGenomics (Lubbock, TX).

### Sequencing

DNA samples were amplified for sequencing at RTLGenomics (Lubbock, TX) in a two-step process. The forward primer was constructed with (5'-3') the Illumina i5 sequencing primer (TCGTCCGACGTCAGATGTG-TATAAGAGACAG) and the 28F primer (GAGTTT-GATCNTGGCTCAG). The reverse primer was constructed with (5'-3') the Illumina i7 sequencing primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA G) and the 388R primer (TGCTGCCTCCCGTAGG AGT). This primer set amplifies the first and second hypervariable regions (V1–V2) of bacterial 16S rRNA.

Amplifications were performed in 25  $\mu$ L reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, CA), 1  $\mu$ L of each 5  $\mu$ M primer, and 1  $\mu$ L of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, CA) under the following thermal profile: 95  $^{\circ}$ C for 5 min, then 35 cycles of 94  $^{\circ}$ C for 30 s, 54  $^{\circ}$ C for 40 s, 72  $^{\circ}$ C for 1 min, followed by one cycle of 72  $^{\circ}$ C for 10 min and 4  $^{\circ}$ C hold.

Products from the first stage amplification were added to a second PCR based on qualitatively determined concentrations. Primers for the second PCR were designed based on the Illumina Nextera PCR primers as follows: Forward - AATGATACGGCGACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC and Reverse - CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG. The second stage amplification was run the same as the first stage except for 10 cycles.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was size selected in two rounds using SPRIselect (BeckmanCoulter, Indianapolis, IN) in a 0.7 ratio for both rounds. Size selected pools were then run on a Fragment Analyzer (Advanced Analytical, Ankeny, IA) to assess the size distribution, quantified using the Qubit 3.0 fluorometer (Life Technologies), and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, CA) 2  $\times$  300 flow cell at 10 pM and sequenced at RTLGenomics. Then, the resulting sequences were run through RTLGenomics' standard microbial diversity analysis pipeline which resulted in operational taxonomic units (OTUs) and taxonomic classifications (17).

### Bioinformatics analysis

All sequencing reads were run through Research and Testing Laboratory's (RTLGenomics, Lubbock, TX, USA) standard microbial analysis pipeline. The data analysis pipeline consisted of two major stages, the denoising and chimera detection stage and the microbial diversity analysis stage. During the denoising and chimera detection stage, denoising was performed using various techniques to remove short sequences, singleton sequences, and noisy reads using the USEARCH (18) and UPARSE (19) algorithms. With the low-quality reads removed, chimera detection was performed to aid in the removal of chimeric sequences using the UCHIME chimera detection software in *de novo* mode (19). Lastly, the remaining sequences were then corrected per-base to help remove errors in sequencing.

During the diversity analysis stage, all samples were assembled into OTU clusters at 97% identity using the UPARSE (Edgar 2013) algorithm and then globally aligned using the USEARCH (Edgar 2010) global algorithm against a database of high-quality 16S rRNA bacterial gene sequences from GenBank, compiled by RTL, to determine taxonomic classifications. After OTU selection was performed, a phylogenetic tree was constructed in Newick format from a multiple sequence alignment of the OTUs done in MUSCLE (20, 21) and generated in FastTree (22, 23). Based on the above OTU table and taxonomy file generated, the bacteria were classified at the appropriate taxonomic levels using trimmed taxa which take confidence values into account at each taxonomic

level. The percentage of sequences assigned to each bacterial phylogenetic level was individually analyzed for each pooled sample providing relative abundance information within and among the individual samples.

### Aerotolerance categorizing

After species identification, we labeled each species with their respective aerotolerance (aerobe, anaerobe, facultative, and microaerophilic bacteria) based on an in-house database at RTLGenomics, Research and Testing Laboratory. This database was compiled based on information from the International Journal of Systemic and Evolutionary Microbiology. We then normalized the relative abundance of species in each aerotolerance category to reflect only the characterized OTUs. In this section, we report this adjusted relative abundance. For purposes of this work, anaerobe refers to obligate anaerobes while facultative refers to facultative anaerobes.

### Statistical analysis

Data were summarized as median (interquartile range) and frequency (percentage). p-values for differences between groups (Abscess vs NSTI Survivor vs NSTI Non-Survivor) in medians were calculated using Kruskal–Wallis equality-of-populations rank test, and subsequently Dunn's test was used for pairwise comparisons when appropriate. Since some cells presented small frequencies, p-values for categorical outcomes, including wound data, were calculated using Fisher's exact test. Bonferroni's method was used to adjust multiple pairwise comparisons. Significance level was set at 0.05.

## RESULTS

### Patient characteristics

The demographics of our cohort display the same trends as have been described in other studies (24–26) (Table 1). NSTI patients, both survivors and non-survivors, were older than abscess patients (p-value 0.029 and 0.005, respectively). NSTI were similar to abscess patients in BMI, but the BMI of NSTI non-survivors was higher than NSTI survivors (p-value 0.001). Medical history revealed that both NSTI survivors and non-survivors had more co-morbidities compared to abscess patients (p-value 0.026 and 0.0030, respectively). Diabetes mellitus was much more common in NSTI patients compared to abscess patients (p = 0.037 for survivors and p < 0.001 for non-survivors). Additionally, more NSTI non-survivors had hypertension and obesity (defined by BMI > 30) than did abscess patients (p-values 0.030 and 0.009, respectively). However, our NSTI cohort were more extreme in increased BMI and number of co-morbidities compared to previous studies (4, 27), which reflects the West Texas demographic. NSTI patients had an extensive antibiotic history (Table S1), both prior

**Table 1.** Demographics, co-morbidities, and clinical course of our three patient cohorts (abscess patients, NSTI survivors, and NSTI non-survivors)

	Abscess (n = 20)	NSTI survivor (n = 23)	NSTI non-survivor (n = 5)	p-value	Adj. Pairw. p- value	
					S vs A	NS vs A
Age (years), median (IQR)	34.5 (23–50.5)	49 (38–56)	56 (53–63)	<b>0.008</b>	<b>0.029</b>	<b>0.005</b>
BMI, median (IQR)	27.5 (24.7–31.9)	30.7 (25.8–39.9)	46.6 (44.7–51.7)	<b>0.005</b>	0.164	<b>0.001</b>
Sex, n (%)				0.068		
Female	12 (60)	10 (43.5)	5 (100)			
Male	8 (40)	13 (56.5)	0 (0)			
Ethnicity, n (%)				0.271		
White	12 (60)	10 (43.5)	3 (60)			
Hispanic	4 (20)	11 (47.8)	2 (40)			
Black	1 (5)	2 (8.7)	0 (0)			
Other	3 (15)	0 (0)	0 (0)			
DM, n (%)				<b>0.000</b>	<b>0.037</b>	<b>&lt;0.001</b>
Controlled	2 (10)	3 (13)	4 (80)			
Uncontrolled	0 (0)	6 (26.1)	1 (20)			
Smoker, n (%)	5 (25)	11 (47.8)	2 (40)	0.320		
EtOH abuse, n (%)	0 (0)	4 (17.4)	1 (20)	0.117		
CAD, n (%)	2 (10)	3 (13)	0 (0)	1.000		
Hypertension, n (%)	7 (35)	8 (34.8)	5 (100)	<b>0.022</b>	1.000	<b>0.030</b>
COPD, n (%)	1 (5)	2 (8.7)	1 (20)	0.436		
CKD, n (%)	0 (0)	1 (4.3)	1 (20)	0.199		
CHF, n (%)	0 (0)	0 (0)	1 (20)	0.104		
Obesity, n (%)	5 (25)	13 (56.5)	5 (100)	<b>0.004</b>	0.125	<b>0.009</b>
Total # comorb, med (IQR)	0 (0–2)	2 (1–3)	5 (3–5)	<b>0.001</b>	<b>0.026</b>	<b>0.030</b>
LOS (total) median (IQR)	0 (0–0)	14 (8–18)	22 (21–26)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
LOS (ICU), median (IQR)	0 (0–0)	6 (1–14)	22 (20–26)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>

Overall, NSTI non-survivors were the oldest, had the most co-morbidities, and longest length of stay. p-values for differences in continuous outcomes were calculated using Kruskal–Wallis equality-of-populations rank test, and Dunn's test for pairwise comparisons. p-values for categorical outcomes were calculated using Fisher's exact test. Bonferroni's method was used to adjust pairwise comparisons. A, abscess patient; CAD, coronary artery disorder; CHF, congestive heart failure; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; EtOH, ethanol; LOS, length of stay; NS, NSTI non-survivor; S, NSTI survivor. Bold values were significantly different between NSTI non-survivors and the other categories.

to sampling and throughout their hospital stay. On the other hand, abscess patients (data not shown) took little to no antibiotics prior to their clinic visits and were rarely on more than one antibiotic throughout their infection course.

### Sites of infections

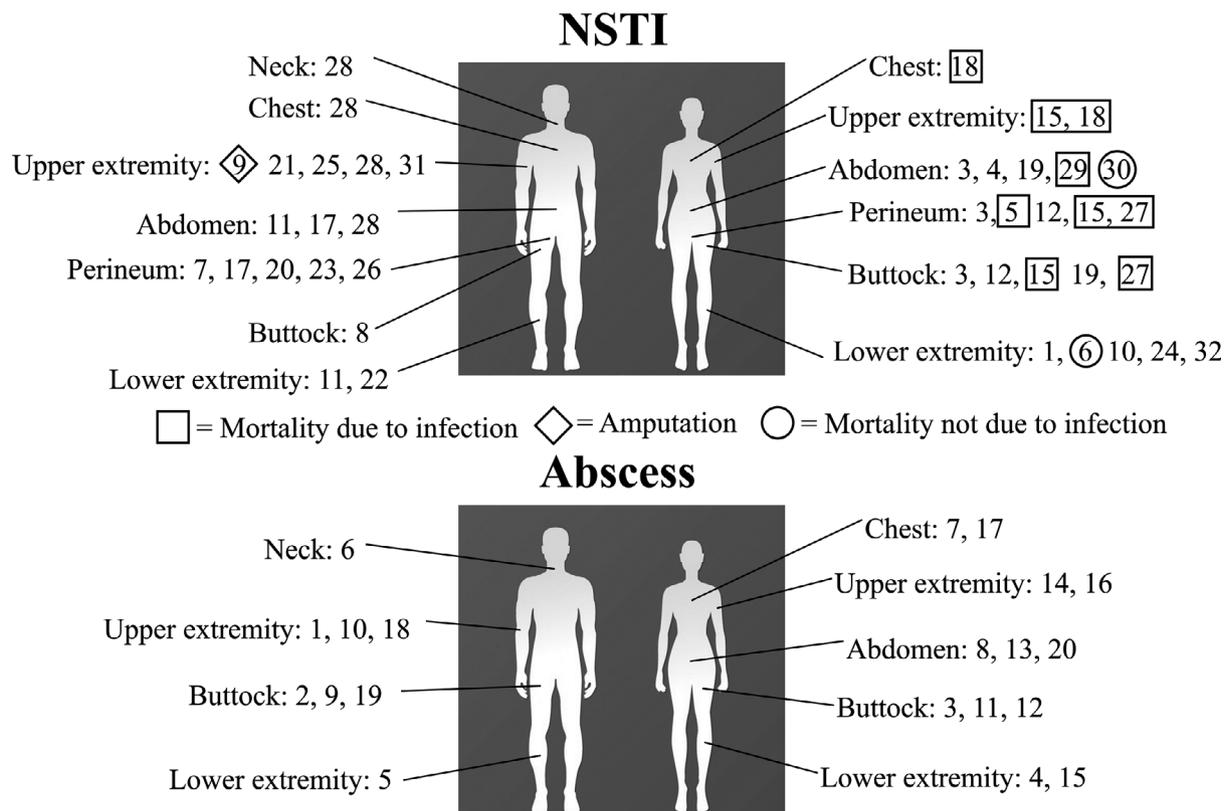
Among NSTI patients, the wound locations were mostly evenly distributed (Table S2, Fig. 1A). All five deaths from NSTIs were among women. Although men and women had NSTIs in the perineum at similar rates (five perineum NSTIs each), women died from their perineal NSTIs more frequently than men (3 vs 0), and an infection of the perineum was significantly associated with NSTI over an abscess (p-values 0.020 for survivors and 0.009 for non-survivors). Wound sizes did not appear to be associated with mortality. For example, patient 28 suffered a NSTI originating in the oral cavity and spread down his neck and eventually covered his chest, abdomen, and part of his upper arm and he survived the infection. In

contrast, patients 5 and 29 had their infections in a single area (perineum and abdomen, respectively), and they both died from their infections. The wounds of our 20 abscess patients were in slightly different places (Fig. 1B). None of our male patients had any chest, abdomen, or perineum abscesses and our female patients did not have any perineum abscesses.

### Microbiology findings

Our NSTI cohort had 5 mortalities and 20 survivors, excluding 3 patients whose wound samples did not yield enough DNA for 16S rRNA sequencing analysis (16S). Abscess wounds are not routinely cultured, so a concordance between culture and 16S rRNA analysis was not possible.

We first compared the culture results as reported by our Clinical Microbiology Laboratory (CML) to our 16S data for NSTI patients (Table S3). We found that 13 of our 20 (65%) NSTI survivors and 3 of our 5 (60%) NSTI non-survivors had any concordance between the culture and the top five most



**Fig. 1.** Visual representation of wounds by sex and location. Each number is associated with our study participant code number. For both NSTI and abscess patients, the wound location was widespread and evenly distributed among men and women. (A) NSTI wounds. Square around the number indicates mortality due to the NSTI, diamond indicates an amputation, and circle indicates mortality not due to the NSTI. (B) Abscess wounds. All abscess patients survived with no notable morbidities.

abundant species detected by 16S. However, this concordance was not always meaningful. For example, patient 9's culture results indicated an organism of the *Streptococcus viridans* group, *Eikenella corrodens*, and normal flora. Our 16S results additionally listed *Streptococcus anginosus* as an abundant organism, but an unknown *Prevotella* species was even more abundant. This led us to look at how many of our patients had a concordance between their culture findings and the most abundant species found by 16S. We found that seven NSTI survivors (35%) and one NSTI non-survivor (20%) met this criterion. Of the seven NSTI survivors without concordance between culture and 16S, five (71.4%) were because 16S identified an anaerobe that was not identified by culture. This was also the case for both of the NSTI non-survivors without concordance between culture and 16S. Both 16S and CML were able to detect anaerobes in eight patients, including two non-survivors. In total, CML made the claim of 'no anaerobes' in their reports for 11 of our NSTI patients, 2 of whom were NSTI non-survivors. In those cases, the

anaerobe that was missed was most commonly *Prevotella* species, followed by *Bacteroides*, *Anaerococcus*, *Peptoniphilus*, and *Porphyromonas* species.

Next, we wanted to see if any specific genus was indicative of a mortality or development of NSTI. Using only the data from NSTI mortalities (five patients) we searched for any genus that was abundant in at least one mortality (>10% abundance) and was found in at least three of the five mortalities. This focused our analysis to only genera that were commonly present and abundant in NSTIs causing mortality. We found five genera of interest: *Enterococcus*, *Porphyromonas*, *Peptoniphilus*, *Bacteroides*, and *Prevotella*. Next, we examined the involvement of these genera in our other patient cohorts, NSTI survivors and abscess patients. We found that the genera *Enterococcus*, *Porphyromonas*, and *Bacteroides* were more commonly present in NSTI mortalities as compared to the other groups (Table 2), although results were only significant for *Enterococcus*. *Prevotella* species were frequently present in all three groups, but were found in all 5 NSTI mortalities, vs 18/20 NSTI

**Table 2.** Proportion of each patient group with genera of interest

	Abscess (n = 18)	NSTI survivor (n = 20)	NSTI non-survivor (n = 5)	p-value	Adj. Pairw. p-value	
					A vs NS	S vs NS
<i>Prevotella</i>	12 (66.7)	18 (90)	5 (100)	0.152		
<i>Bacteroides</i>	3 (16.7)	5 (25)	3 (60)	0.169		
<i>Peptoniphilus</i>	12 (66.7)	10 (50)	4 (80)	0.418		
<i>Porphyromonas</i>	7 (38.9)	9 (45)	4 (80)	0.297		
<i>Enterococcus</i>	2 (11.1)	1 (5)	3 (60)	0.017	0.096	<b>0.032</b>

Bold values were significantly different between NSTI non-survivors and the other categories.

survivors and 12/18 abscess patients. When looking at NSTI patients alone, many of these genera were common in both mortalities and survivors, but the majority of the patients with these genera survived (Fig. 2). While this observation is skewed by the fact that we had a much higher population of NSTI survivors than non-survivors, NSTI non-survivors were more afflicted with these species compared to NSTI survivors. This is especially true for *Enterococcus*. Although present in only 4 of our 25 NSTI patients, 3 of those patients died from their infection, indicating that *Enterococcus* was associated with a higher mortality rate ( $p = 0.032$ ).

We also looked at the bacterial diversity of the wounds in the different patient groups using the Shannon index of diversity, which takes into account both richness and evenness of the bacterial species, and the Chao index, which only takes into account richness (Fig. 3 and Table S4). We found that the wounds of NSTI non-survivors trended toward more bacterial diversity compared to abscesses with both indices, although results were not significant.

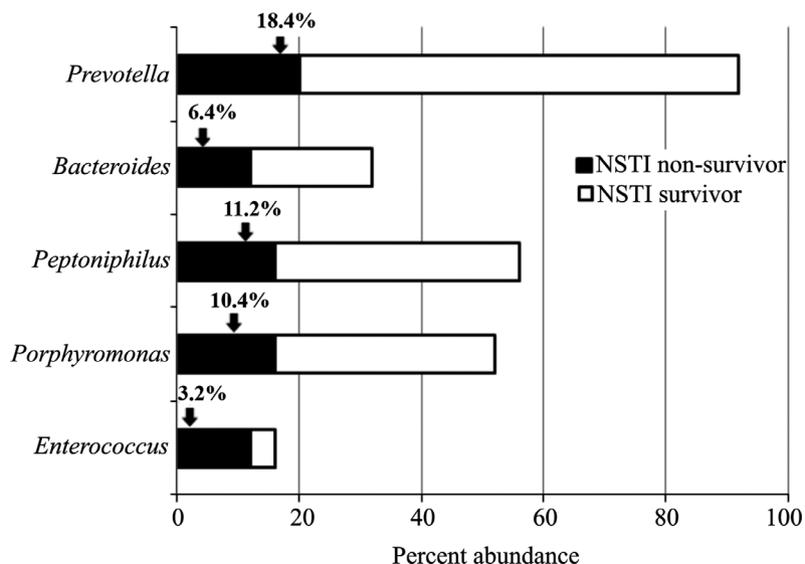
Next, we wanted to see if there were differences in bacterial aerotolerance among the different infection categories. The relative abundance data are reported as a median (interquartile range) proportion of all identified OTUs in each wound belonging to each aerotolerance category. We found that among NSTI non-survivors, anaerobes contributed to the vast majority of the wound consortium [0.9 (0.8–1), Fig. 4A, Table S4]. Facultative anaerobes only contributed 0 (0–0.2) and aerobes and microaerophilic bacteria contributed even smaller amounts. This is contrasted with abscess patients, where anaerobes were much less abundant [0.2 (0–1)]. The wounds of NSTI survivors fell in the middle [0.8 (0–1)]. While the proportion of facultative anaerobes in both NSTI survivors and abscess patients was higher [0.1 (0–0.9) and 0.6 (0–1), respectively] than in NSTI non-survivors, the results were not significant. To note: results of NSTI survivors and abscess patients were highly variable, but the results of NSTI non-survivors were tightly clustered.

Overall, we found that the bacterial populations in all five of our NSTI non-survivors (100%) had at least a 70% abundance of anaerobes, whereas fewer NSTI survivors and abscess patients met this criterion (50% NSTI survivors and 38.9% abscess patients,  $p$ -value 0.028 comparing NSTI non-survivors with abscess patients Fig. 4B). For our patient cohort, the criteria of >70% relative abundance of anaerobes represented a 60% sensitivity and 66% specificity of getting a NSTI and a 100% sensitivity and 50% specificity of dying from the NSTI.

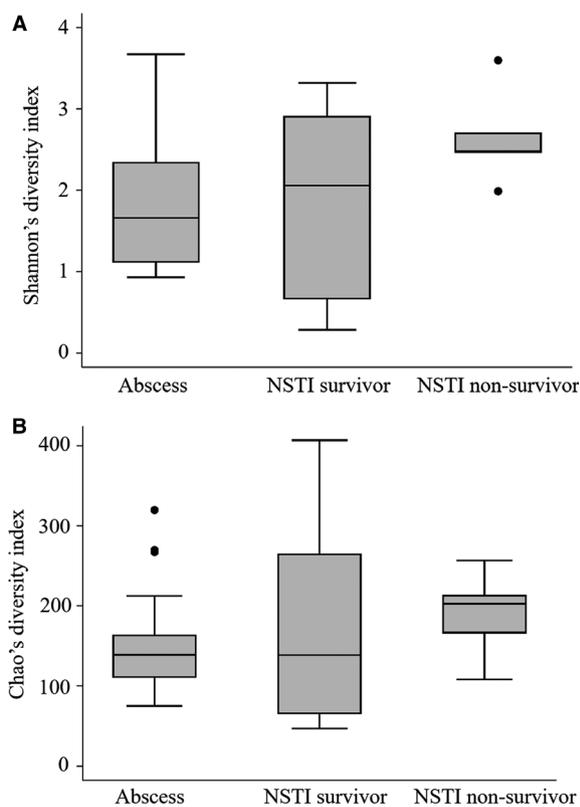
Lastly, we grouped our wound bacteria based on their gram designation, and found that both NSTI non-survivors and survivors had significantly more gram negative bacteria in their wounds compared to abscess patients ( $p$ -values are 0.045 and 0.025, respectively, Fig 5, Table S4). Administration of antibiotics can have an effect on the microbes found, especially since many of the first-line antibiotics used in NSTIs tend to target gram positives. So it can be argued that administration of a gram positive targeting antibiotic, such as Vancomycin, skews our results toward gram negative bacteria. However, we found that of our 25 NSTI patients with molecular results, only two were not administered Vancomycin (Table S1). The wounds of these two patients had 86% and 78% gram negative bacteria, respectively. On the contrary, our patients with the highest proportion of gram positive bacteria, (99.84% and 99.88%) both were administered Vancomycin before sample collection. Therefore, antibiotic administration alone does not appear to cause the high distribution of gram negative bacteria we observe in NSTI survivors and non-survivors.

## DISCUSSION

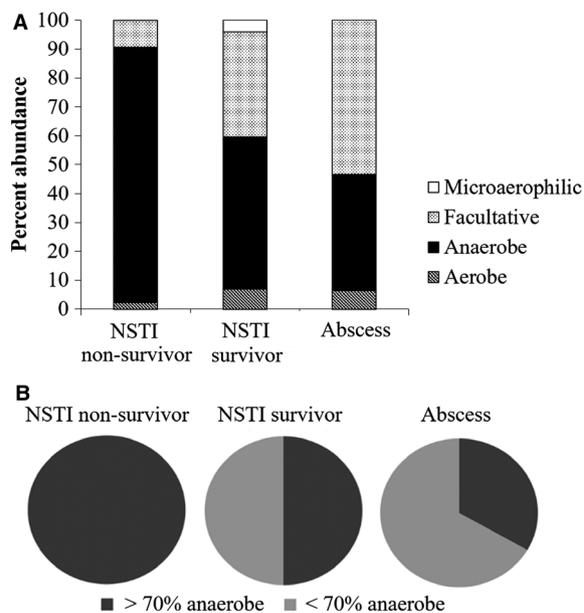
The NSTIs are increasing in incidence (4, 28) and despite aggressive treatment, cause a high mortality rate. The specific pathophysiology of NSTIs is not well understood. Part of the reason for this gap in knowledge could be a lack of fundamental information regarding the microorganisms involved in



**Fig. 2.** Percentage of patients with the specified genus who were NSTI non-survivors or survivors. Arrow indicates the 20% mark of the population with each genus of bacteria (20% of our NSTI patients were non-survivors).



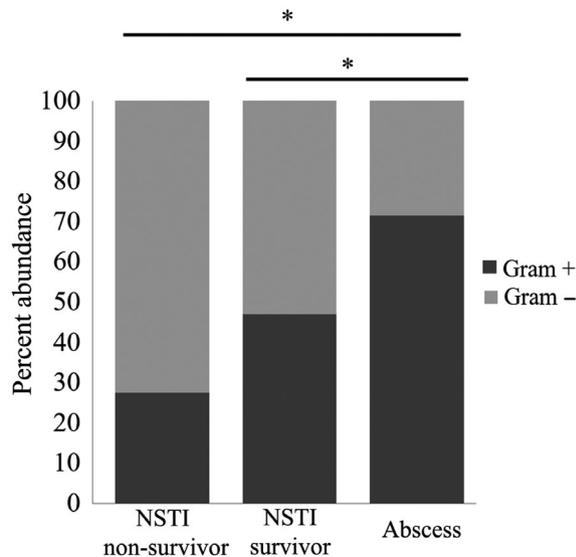
**Fig. 3.** Shannon and Chao indices of wound diversity.



**Fig. 4.** Aerotolerance of NSTI survivors, NSTI non-survivors, and abscess patients. (A) Mean percent distribution of bacteria based on aerotolerance and on patient populations. (B) Proportions of patients in each group with wound microbiota composed of >70% obligate anaerobes. p-value = 0.028 comparing wounds NSTI non-survivors to abscess patients. p-values were calculated using Fisher's exact test.

NSTIs. There have been numerous reports on the microbiology of NSTIs (4, 25–27, 29–31), but they have almost always used data obtained by

traditional culture methods. Furthermore, many institutions report culture-negative results in a rapidly progressive wound bed (4, 24, 26), further



**Fig. 5.** Gram designation of NSTI survivors, NSTI non-survivors, and abscess patients. \* $P < 0.05$  should be sufficient.

illustrating the restraints of culture-based diagnostics.

Recently, with the increasing accessibility and affordability of Next Generation Sequencing technology, some institutions have begun using 16S rRNA sequencing to identify bacteria present in various types of infections. For example, one wound care institute implemented 16S rRNA sequencing to their diagnostic process and compared the microbial and wound healing results before and after this implementation (32). From the microbiology perspective, they found that 16S rRNA sequencing resulted in much more diversity compared to their culture results and much of this diversity was composed of fastidious growing organisms, including obligate anaerobes. They reported that 16S rRNA sequencing resulted in a more accurate diagnosis of the microbiology present in wounds, and that this better microbiology report allowed them to reduce broad-spectrum antibiotic usage, resulting in more affordable treatment. They also reported that wounds closed faster because of the targeted therapy.

Molecular diagnostics have also been reported for NSTIs (33). Rudkjobing *et al.* compared results between culture and four different types of molecular diagnostics. They reported that molecular methods and culture agreed with each other generally, but there were cases in which molecular diagnosis caught organisms that culture did not. In two of their patients with similar backgrounds, culture

reported *Bacteroides fragilis* in the first patient but not the second, whereas it was detected in both patients by 454-pyrosequencing. Clinically, the first patient's antibiotic therapy was appropriately supplemented with an anaerobe-specific antibiotic, whereas the second patient's therapy was not. The first patient survived the infection, but the second did not. This difference of antibiotic usage is surely not the only difference between these two patients, but it may have contributed to the death of the second patient, further emphasizing the significance of proper diagnosis and understanding of the microbiology of NSTIs.

However, to our knowledge, we are the first to report 16S rRNA sequencing results as a comparison between NSTIs and abscesses. In this study, we aimed to identify organisms or groups of organisms that potentially shift a wound infection toward the NSTI pathology over the easily treatable abscess pathology. Our sequencing results have allowed us to highlight groups of bacteria not normally associated with NSTIs, and specifically NSTI mortalities. We found that when there was no concordance between culture and sequencing, it was most commonly because culture failed to detect obligate anaerobes. We saw that of the five genera most commonly detected in NSTI non-survivors by 16S rRNA sequencing (*Prevotella*, *Bacteroides*, *Peptoniphilus*, *Porphyromonas*, and *Enterococcus*), four were obligate anaerobes. This is contrary to previous reports, which emphasize *Staphylococcus* and *Streptococcus* species as most commonly isolated in NSTIs (34). *Bacteroides* species were commonly reported in NSTIs, possibly because of their high tolerance to oxygen compared to other genera we report here (35). *Enterococcus*, however, was the genus significantly associated with mortality and was rare in NSTI survivors and abscess patients, and this has been corroborated by several other reports (4, 31, 36, 37).

We also saw a large difference in the relative abundance of anaerobes in NSTI non-survivors compared to survivors and abscess patients. However, we showed that wounds with more than 70% relative abundance of anaerobes were significantly associated with mortality. All of our NSTI mortalities were obese and obesity has been linked to tissue hypoxia, especially in the adipose and non-adipose cells of fat tissue (38). This tissue hypoxia, along with the facultative anaerobes found within the sample, could be allowing obligate anaerobes to survive in the wound environment. We postulate that the persistence of these obligate anaerobes may be contributing to the mortalities in our NSTI cohort. Lastly, we showed that both NSTI non-survivors and survivors had significantly more gram negative bacteria in their wounds compared to abscess patients. This is

contrary to what has been previously reported. While some gram negative rods, such as *Escherichia coli* and *Bacteroides fragilis* were highlighted in previous studies (7, 29, 39, 40), the majority of studies associate NSTIs with gram positive bacteria, such as *Staphylococcal* and *Streptococcal* species (34). Our NSTI cohorts had a higher relative abundance of obligate anaerobes, such as *Bacteroides* and *Prevotella* species, which are mainly gram negative bacteria. On the other hand, our abscess cohort had *Staphylococcus* and *Streptococcus* species, which increased their gram positive relative abundances.

This study does have some weaknesses. First, by using the 16S rRNA sequencing, we are unable to detect fungi in our samples because they do not have a 16S rRNA gene. However, not being able to detect fungi in our samples does not negate the fact that clinical laboratory was unable to detect many of the bacterial species found in our study. Our claim, that the clinical laboratory is insufficient at identifying microbial species, is still supported by our data. Second, our study identified all the bacteria in the wound sample without characterizing them as colonizing or pathogenic bacteria. To address this, we should first revisit the concept of colonizing vs pathogenic bacteria, and if this is a valid distinction. A reasonable method of distinguishing between a colonizer and a pathogen is the absence or presence of certain virulence factors important in causing disease. However, *S. aureus*, which possesses a wide variety of virulence factors and is capable of causing a multitude of infections, is also capable of colonizing the nares of certain individuals (41). Is *S. aureus*, then, a colonizer, or a pathogen? Over the past 20 years, a theory known as the Damage-Response Framework has surfaced and argues that designations of microbes such as 'pathogens' and 'commensals' are irrelevant (42, 43). The reality is that most bacteria can cause infection in the right circumstance. Significant for our study, we identified many organisms in the wounds of NSTIs and abscesses via 16S rRNA sequencing, and while some of them may not be involved in the infection process, we cannot label all bacteria without known virulence factors, or that have thus far not been implicated in diseases, as irrelevant.

Often when presented with sequencing data, our clinical colleagues, especially infectious disease (ID) physicians often reply, 'It's great you're finding all these bacteria in the wound, but what would you like us to do about it?' This is an important question because often times for NSTIs, ID has already prescribed broad-spectrum antibiotics. What more can be done? In this case, it is important to note that anaerobes are becoming increasingly resistant to antibiotics. One institution has shown increasing rates of resistance for *Bacteroides fragilis* to

clindamycin and moxifloxacin (44, 45), and other anaerobes are also increasingly antibiotic resistant (46). In light of the important role anaerobes likely have in NSTIs and the increasing resistance to anaerobes, it may be wise to increase anaerobe coverage. Additionally, 100% of our wound samples were polymicrobial. Interactions between microbes can influence their growth, pathogenesis and antibiotic tolerance, so gaining a better understanding of polymicrobial interactions in NSTIs may be key to stopping these infections in the future.

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## REFERENCES

1. Dryden MS. Complicated skin and soft tissue infection. *J Antimicrob Chemother* 2010;65(Suppl 3):iii35–44.
2. May AK. Skin and soft tissue infections. *Surg Clin North Am* 2009;89:403–20, viii.
3. Bonne SL, Kadri SS. Evaluation and management of necrotizing soft tissue infections. *Infect Dis Clin North Am* 2017;31:497–511.
4. Bernal NP, Latenser BA, Born JM, Liao J. Trends in 393 necrotizing acute soft tissue infection patients 2000–2008. *Burns* 2012;38:252–60.
5. Kobayashi SD, Malachowa N, DeLeo FR. Pathogenesis of *Staphylococcus aureus* abscesses. *Am J Pathol* 2015;185:1518–27.
6. Johnson RC, Ellis MW, Schlett CD, Millar EV, LaBreck PT, Mor D, et al. Bacterial etiology and risk factors associated with cellulitis and purulent skin abscesses in military trainees. *PLoS One* 2016;11:e0165491.
7. Childers BJ, Potyondy LD, Nachreiner R, Rogers FR, Childers ER, Oberg KC, et al. Necrotizing fasciitis: a fourteen-year retrospective study of 163 consecutive patients. *Am Surg* 2002;68:109–16.
8. Elliott DC, Kufera JA, Myers RA. Necrotizing soft tissue infections. Risk factors for mortality and strategies for management. *Ann Surg* 1996;224:672–83.
9. Harbrecht BG, Nash NA. Necrotizing soft tissue infections: a review. *Surg Infect (Larchmt)* 2016;17:503–9.
10. Sood S, Nerurkar V. Fatal necrotizing soft tissue infection by *Aeromonas hydrophila*. *J Clin Diagn Res* 2014;8:Dd06-7.
11. Butterworth SA, Murphy JJ. Necrotizing soft tissue infections—are they different in healthy vs immunocompromised children? *J Pediatr Surg* 2006;41:935–9.
12. Hua C, Sbidian E, Hemery F, Decousser JW, Bosc R, Amathieu R, et al. Prognostic factors in necrotizing soft-tissue infections (NSTI): a cohort study. *J Am Acad Dermatol* 2015;73:1006–12. e8.

13. Thurlow LR, Joshi GS, Richardson AR. Virulence strategies of the dominant USA300 lineage of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA). *FEMS Immunol Med Microbiol* 2012;65:5–22.
14. Diekema DJ, Richter SS, Heilmann KP, Dohrn CL, Riahi F, Tendolkar S, et al. Continued emergence of USA300 methicillin-resistant *Staphylococcus aureus* in the United States: results from a nationwide surveillance study. *Infect Control Hosp Epidemiol* 2014;35:285–92.
15. Lemmens N, van Wamel W, Snijders S, Lesse AJ, Faden H, van Belkum A. Genomic comparisons of USA300 *Staphylococcus aureus* colonizing the nose and rectum of children with skin abscesses. *Microb Pathog* 2011;50:192–9.
16. Jorth P, Turner KH, Gumus P, Nizam N, Buduneli N, Whiteley M. Metatranscriptomics of the human oral microbiome during health and disease. *MBio* 2014;5:e01012-14.
17. Javan GT, Finley SJ, Can I, Wilkinson JE, Hanson JD, Tarone AM. Human thanatomicrobiome succession and time since death. *Sci Rep* 2016;6:29598.
18. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26:2460–1.
19. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 2011;27:2194–200.
20. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;32:1792–7.
21. Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 2004;5:113.
22. Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 2009;26:1641–50.
23. Price MN, Dehal PS, Arkin AP. FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS ONE* 2010;5:e9490.
24. Jabbour G, El-Menyar A. Pattern and predictors of mortality in necrotizing fasciitis patients in a single tertiary hospital. *World J Emerg Surg* 2016;11:40.
25. Kha P, Colot J, Gervolino S, Guerrier G. Necrotizing soft-tissue infections in New Caledonia: epidemiology, clinical presentation, microbiology, and prognostic factors. *Asian J Surg* 2016;40:290–4.
26. Wang JM, Lim HK. Necrotizing fasciitis: eight-year experience and literature review. *Braz J Infect Dis* 2014;18:137–43.
27. Keung EZ, Liu X, Nuzhad A, Adams C, Ashley SW, Askari R. Immunocompromised status in patients with necrotizing soft-tissue infection. *JAMA Surg* 2013;148:419–26.
28. Soltani AM, Best MJ, Francis CS, Allan BJ, Askari M, Panthaki ZJ. Trends in the incidence and treatment of necrotizing soft tissue infections: an analysis of the National Hospital Discharge Survey. *J Burn Care Res* 2014;35:449–54.
29. Glass GE, Sheil F, Ruston JC, Butler PE. Necrotising soft tissue infection in a UK metropolitan population. *Ann R Coll Surg Engl* 2015;97:46–51.
30. Martinschek A, Evers B, Lampl L, Gerngross H, Schmidt R, Sparwasser C. Prognostic aspects, survival rate, and predisposing risk factors in patients with Fournier's gangrene and necrotizing soft tissue infections: evaluation of clinical outcome of 55 patients. *Urol Int* 2012;89:173–9.
31. van Stigt SF, de Vries J, Bijker JB, Mollen RM, Hekma EJ, Lemson SM, et al. Review of 58 patients with necrotizing fasciitis in the Netherlands. *World J Emerg Surg* 2016;11:21.
32. Wolcott RD, Cox SB, Dowd SE. Healing and healing rates of chronic wounds in the age of molecular pathogen diagnostics. *J Wound Care* 2010;19:272–8, 80–81.
33. Rudkjobing VB, Thomsen TR, Xu Y, Melton-Kreft R, Ahmed A, Eickhardt S, et al. Comparing culture and molecular methods for the identification of microorganisms involved in necrotizing soft tissue infections. *BMC Infect Dis* 2016;16:652.
34. Zhao-Fleming H, Dissanaik S, Rumbaugh K. Are anaerobes a major, underappreciated cause of necrotizing infections? *Anaerobe* 2017;45:65–70.
35. Sund CJ, Rocha ER, Tzianabos AO, Wells WG, Gee JM, Reott MA, et al. The *Bacteroides fragilis* transcriptome response to oxygen and H<sub>2</sub>O<sub>2</sub>: the role of OxyR and its effect on survival and virulence. *Mol Microbiol* 2008;67:129–42.
36. Hsiao CT, Weng HH, Yuan YD, Chen CT, Chen IC. Predictors of mortality in patients with necrotizing fasciitis. *Am J Emerg Med* 2008;26:170–5.
37. Moore SA, Levy BH, Prematilake C, Dissanaik S. The prediction predicament: rethinking necrotizing soft tissue infections mortality. *Surg Infect (Larchmt)* 2015;16:813–21.
38. Foti DP, Brunetti A. Editorial: “linking hypoxia to obesity”. *Front Endocrinol* 2017;8:34.
39. Elliott D, Kufera JA, Myers RA. The microbiology of necrotizing soft tissue infections. *Am J Surg* 2000;179:361–6.
40. Magala J, Makobore P, Makumbi T, Kaggwa S, Kalanzi E, Galukande M. The clinical presentation and early outcomes of necrotizing fasciitis in a Ugandan Tertiary Hospital—a prospective study. *BMC Res Notes* 2014;7:476.
41. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG Jr. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev* 2015;28:603–61.
42. Casadevall A, Pirofski LA. The damage-response framework of microbial pathogenesis. *Nat Rev Microbiol* 2003;1:17–24.
43. Pirofski LA, Casadevall A. What is infectiveness and how is it involved in infection and immunity? *BMC Immunol* 2015;16:13.
44. Snyderman DR, Jacobus NV, McDermott LA, Golan Y, Goldstein EJ, Harrell L, et al. Update on resistance of *Bacteroides fragilis* group and related species with special attention to carbapenems 2006–2009. *Anaerobe* 2011;17:147–51.
45. Snyderman DR, Jacobus NV, McDermott LA, Ruthazer R, Golan Y, Goldstein EJ, et al. National survey on the susceptibility of *Bacteroides fragilis* group: report and analysis of trends in the United States from 1997 to 2004. *Antimicrob Agents Chemother* 2007;51:1649–55.
46. Hastej CJ, Boyd H, Schuetz AN, Anderson K, Citron DM, Dzink-Fox J, et al. Changes in the antibiotic susceptibility of anaerobic bacteria from 2007–2009 to 2010–2012 based on the CLSI methodology. *Anaerobe* 2016;42:27–30.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Antibiotic usage of NSTI patients.

**Table S2.** Wounds by sex and location summary.

**Table S3.** Comparison of traditional culture results to 16S sequencing results.

**Table S4.** Microbiology summary.