HAYSTAC: A Bayesian framework for robust and rapid species identification in high-throughput sequencing data

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Identification of specific species in metagenomic samples is critical for several key applications, yet many tools available require large computational power and are often prone to false positive identifications. Here we describe High-AccuracY and Scalable Taxonomic Assignment of MetagenomiC data (HAYSTAC), which can estimate the probability that a specific taxon is present in a metagenome. HAYSTAC provides a user-friendly tool to construct databases, based on publicly available genomes, that are used for competitive reads mapping. It then uses a novel Bayesian framework to infer the abundance and statistical support for each species identification, and provide per-read species classification. Unlike other methods, HAYSTAC is specifically designed to efficiently handle both ancient and modern DNA data, as well as incomplete reference databases, making it possible to run highly accurate hypothesis-driven analyses (*i.e.*, assessing the presence of a specific species) on variably sized reference databases while dramatically improving processing speeds. We tested the performance and accuracy of HAYSTAC using simulated Illumina libraries, both with and without ancient DNA damage, and compared the results to other currently available methods (*i.e.*, Kraken2/Braken, MALT/HOPS, and Sigma). HAYSTAC identified fewer false positives than both Kraken2/Braken and MALT in all simulations, and fewer than Sigma in simulations of ancient data. It uses less memory than Kraken2/Braken as well as MALT both during database construction and sample analysis. Lastly, we used HAYSTAC to search for specific pathogens in two published ancient metagenomic datasets, demonstrating how it can be applied to empirical datasets. HAYSTAC is available from `https://github.com/antonisdim/HAYSTAC`

metagenomics | taxonomic classification | pathogens | ancient DNA

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Introduction

Metagenomics allows high-throughput sequencing of complex microbial communities that may not be possible to grow in a laboratory. Accurate identification of specific species within a complex community is critical for metagenomic applications in a wide variety of fields, including medicine, microbiome sciences, environmental sciences, biosurveillance, and biomolecular archaeology. Confidently assessing the presence of individual species is also crucial for taxonomic profiling (Gonzalez et al., 2016), inferring genomic evolution and phylogenetics (Tett et al., 2019), tracing disease outbreaks (Ahn et al., 2015), generating diagnostics from biofluids (Wilson et al., 2019) and studying ancient pathogens (Spyrou et al., 2019).

Recent studies of ancient pathogens based on high-throughput sequencing technology have provided unique insights into the evolutionary history of major human pathogens. These include the etiologic agents of both the Justinian and Black Death plagues (*Yersinia pestis*; (Bos et al., 2011; Schuenemann et al., 2011; Spyrou et al., 2016; Rasmussen et al., 2015)), tuberculosis (the *Mycobacterium tuberculosis* complex; (Pósa et al., 2015; Müller et al., 2014)), syphilis (*Treponema pallidum*; (Schuenemann et al., 2018; Arora et al., 2016)) and smallpox (*Variola major* and *Variola minor*; (Mühlemann et al., 2020)).

Accurately identifying specific microbial species from ancient metagenomes, however, can be challenging for numerous reasons. Firstly, short read sequences originating from closely related species may be difficult to distinguish from each other. In addition, the species of interest may have low abundance in the sample, or the genomes in the reference database may not be representative of the strain present in a sample (Warinner et al., 2017). Contamination from laboratory environment or reagents may also obscure identification (Davis et al., 2018). Identifying specific species within ancient metagenomes is further complicated by issues inherent to ancient DNA, including short molecules (typically between 30–60 bp (Kistler et al., 2017)), post-mortem miscoding lesions (e.g., cytosine deamination), contamination from soil and sediment bacteria in the burial environment, sample handling, and storage (Mann et al., 2018).

When present in ancient metagenomes, pathogen DNA is

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usually found in low abundance (Warinner et al., 2017), and researchers often initially screen many samples before deciding which libraries should be more deeply sequenced in order to obtain whole genome data, or be further processed using targeted capture techniques (Borówka et al., 2019; Key et al., 2017; Spyrou et al., 2019). Screening for pathogens is typically performed by shallow shotgun sequencing followed by *in silico* analyses of short read data using taxonomic classifiers. Following screening, libraries with a positive identification for a species of interest are then more deeply sequenced and investigated, with or without targeted capture (Spyrou et al., 2019). Confident species identification from metagenomic screening data is therefore critical for studies of ancient pathogens.

Tools for detecting microbial species from metagenomes include metagenomic classifiers that report taxonomic relative abundances (Ye et al., 2019), such as Kraken2 (Wood and Salzberg, 2014; Wood et al., 2019) and the Megan Alignment Tool (MALT) (Vågene et al., 2018), as well as species identification pipelines that classify individual reads, such as Sigma (Ahn et al., 2015), Strain Prediction and Analysis using Representative Sequences (SPARSE) (Zhou et al., 2018), Bayesian Reestimation of Abundance with KrakEN (Bracken) (Lu et al., 2017), and Heuristic Operations for Pathogen Screening (HOPS, specifically designed for ancient DNA) (Hübler et al., 2019). The metagenomic classifiers Kraken2 and MALT provide a good starting point for exploratory studies, especially when it is not known which pathogens may be present (Naccache et al., 2014). These tools, however, can be slow, require a large amount of RAM (Hübler et al., 2019; Vågene et al., 2018), and are prone to false positive identifications of low abundance species (Velsko et al., 2018). The species identification pipelines Sigma (Ahn et al., 2015) and SPARSE (Zhou et al., 2018) are based on more sophisticated statistical models but they require alignment to a large database, which can be slow (Zhou et al., 2018), and do not provide statistical support for individual species identifications.

To address these issues, we developed High-AccuracY and Scalable Taxonomic Assignment of MetagenomiC data (HAYSTAC - `https://github.com/antonisdim/HAYSTAC`), a light-weight, fast, and user-friendly species identification tool. HAYSTAC evaluates the presence of a particular species of interest in a metagenomic sample, and provides statistical support for the species assignment. Our method is designed to estimate the probability that a specific taxon is present in a metagenomic sample given a set of sequencing reads and a database of reference genomes. We first derive the probability that the set of reads has originated from a single species (single source identification). This is useful, for example, for taxonomic identification of unknown source material (e.g. (Prendergast et al., 2017)). We then expand this method to identify multiple species in a metagenomic sample (multiple source identification).

To apply HAYSTAC, the user begins by constructing a database from publicly available genomes via a user-friendly automated process. Sample reads are then mapped to all reference genomes in the database using a Bowtie2 wrapper (Langmead and Salzberg, 2012). HAYSTAC then applies a novel Bayesian framework to infer abundance and statistical support for each species identification, and provides a per-read classification, allowing for both hypothesis-driven and exploratory analyses. HAYSTAC can build and make use of arbitrarily sized databases, with minimal effects on sensitivity and specificity. This means that HAYSTAC can provide reliable species identifications by aligning reads to a handful of reference genomes (instead of thousands), without misidentifying taxa because of the restricted database size. This feature dramatically improves its performance relative to other methods. Altogether, HAYSTAC provides a robust method to assess the presence of a specific species in a metagenomic sample in a single step without the need to run multiple programs to assess assignment accuracy, such as is required by other methods (*i.e.*, Bracken for Kraken2 (Lu et al., 2017) and HOPS for MALT).

To evaluate HAYSTAC's performance, we applied it to both simulated microbiome datasets and to empirical datasets that were generated from DNA extracts derived from archaeological human bone. We further used HAYSTAC to assess the presence of putative respiratory pathogens in oral microbiome-derived metagenomes from ancient dental calculus. While dental plaque is known to harbor respiratory pathogens (Sumi et al., 2007), few studies to date have reported these species in its mineralized analogue, dental calculus (Warinner et al., 2014; Eerkens et al., 2018), a discrepancy that may reflect identification biases in studies of the latter. Improved identification of ancient respiratory pathobionts (host-associated microbial taxa that may cause disease under specific conditions) has the potential to add to our understanding of pathogen evolutionary dynamics and even aid vaccine development.

Results

Method Overview

HAYSTAC is a metagenomic species identifier that works with arbitrary user defined databases. The database can be built from any combination of an NCBI search query, a user specified list of NCBI accession codes, the RefSeq representative database of prokaryotic species, or a list of user provided reference assemblies. To construct the database, HAYSTAC uses a heuristic method to select a single representative genome per taxon from those available in the user provided input data (Figure 1).

A database-wide index is then built, followed by a competitive alignment (very fast local alignment mode in Bowtie2, with default parameters) of each sample against the full database. This step ensures that reads with no matches in the database are excluded in all subsequent analyses ("Unknown Source" category).

Reads passing this initial inclusion filter are then aligned

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Figure 1. HAYSTAC's workflow. HAYSTAC consists of three main modules: (i) DATABASE, which builds a database of reference genomes from various user input sources; (ii) SAMPLE, which handles downloading sequencing files from the SRA and pre-processing of samples prior to analysis; and (iii) ANALYSE, which performs an analysis of a sample against a database by applying the mathematical model (see methods) for taxonomic abundance estimation.

against every individual genome in the database (with Bowtie2 in end-to-end mode). For each alignment, we count the number of transitions (T_s) and transversions (T_v), as each type of mismatch contributes differently to our statistical model, in order to handle ancient DNA damage. We then compute a posterior probability for each read/taxon alignment pair, using both mismatch count and expected mismatch probability (σ, default of 0.05). Accuracy can be improved by adjusting the mismatch probability (σ) using prior information on intra- and interspecific variability in the specific set of species analysed.

Reads that possess a posterior probability exceeding a user-defined threshold (default of 0.75, Supplemental Figure 5 and 6) are then assigned to a single taxon. Reads with a lower posterior probability are assigned to the "ambiguous source" category. For each taxon, the count of reads passing the posterior probability threshold are used to compute the mean posterior abundance (and 95% confidence interval); *i.e.*, the relative abundance of a taxon in a sample, using a Dirichlet distribution of the sample input data as a whole. A final validation step, for low depth sequencing data, using breadth of coverage is then used to assess if the assigned reads represent a random genome sample or cluster around specific genome areas.

Computational Performance

We assessed the computational performance of HAYSTAC relative to Sigma, Kraken2/Bracken and MALT by measuring peak memory usage (maximum resident set size) and elapsed execution time (wall clock) for each software package on a machine with 48 CPU cores and 392 GB of RAM under controlled conditions. To control for variability in each measurement, we ran five independent replicates of each job, and performed a linear regression across the observed values.

We first assessed performance when building databases containing 10, 100 and 500 species. For all tested sizes, HAYSTAC uses less memory than either MALT or Kracken2/Bracken, while also being faster for the 10 and 100 species databases and only marginally slower than MALT and Kracken2/Bracken for the 500 species database. For all databases HAYSTAC was slower and used more memory than Sigma, which does not require large indices for its alignments (Figure 2; Supplemental Table S9).

We then assessed the performance of these methods when analysing a one million read dataset against the same three database sizes. HAYSTAC uses less memory than Kraken2/Braken and MALT for all database sizes, but it uses more memory and is slower than SIGMA for all database sizes (Figure 2; Supplemental Table S10).

Lastly, we assessed the performance of these methods when analysing datasets of 10 thousand, 100 thousand and 1 million reads, against a database of 500 species (Supplemental Figure 1; Supplemental Table S10). Memory performance was unchanged from the earlier benchmarks, showing that for all methods, peak memory usage scales with database size rather than sample size. With the exception of Kraken2/Bracken, all other methods saw only a modest increase in runtime with larger input sizes, showing that database size also has a substantial impact on overall runtime.

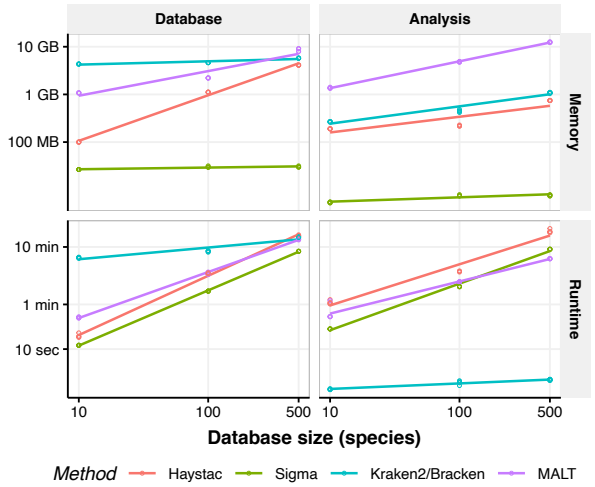

Figure 2. Computational performance of HAYSTAC and other methods. Linear regression of the elapsed time (wall clock) and peak memory usage (maximum resident set size) for a sample of size 1 million reads and reference databases containing either 10, 100 or 500 genomes, each with 5 replicates. When constructing the database, HAYSTAC uses substantially less memory and runs faster than either Kraken2/Bracken or MALT for restricted database sizes. When performing analyses, HAYSTAC uses less memory than either Kraken2/Bracken or MALT, while its runtime was only marginally slower.

Analyses of simulated data sets using RefSeq prokaryotic database

To characterise the sensitivity and specificity of HAYSTAC, we compared its performance to that of three other widely used metagenomic profiling tools: Sigma, Kraken2/Bracken and MALT. We simulated four datasets for these comparisons (Table 1), and then profiled each dataset with all four tools. Each simulated dataset was designed to test a different aspect of performance. For each test, we computed: (i) false positive count (total number of species that were reported yet not present in the simulated sample); (ii) false negative count (total number of species present in the simulation yet were not identified); and (iii) true positive count (total number of species correctly identified).

We first generated a dataset of samples comprising 10 species to quantify the ability of each program to accurately detect species in a simple metagenomic community (hereafter "Simple Microbiome" dataset). We generated two additional sets of profiles for this dataset: one that included modern human DNA ("contaminated") and one that did not ("non-contaminated"). This dataset was created using gargammel (Renaud et al., 2017) by randomly sampling 10 species from a modified RefSeq prokaryote representative genome database (see Methods) and building simulated libraries of one million single-end reads with an equal number of reads per species (100K), both with and without contamination from the human reference genome (25% of the total simulated reads).

All tools correctly identified the 10 species in each simulated sample set (both with and without human contamination; Figure 3). Both Kraken2/Bracken (average of 2.5 species) and MALT (average of 0.5 species) programs reported false positives in the non-contaminated sample set. False positive rates, however, dramatically increased in the sample set with human contamination. HAYSTAC reported the lowest false positive count (5) relative to Sigma (6), Kraken2/Bracken (12.5), and MALT (12.5) (Figure 3). This suggests that HAYSTAC is less prone to false positive identifications caused by human contamination in bacterial reference genomes found in the RefSeq representative prokaryotic database. All false positive identification (accross all programs) involved species whose RefSeq genomes are known to contain human DNA contamination (Breitwieser et al., 2019) (Supplemental Table S7). This result strongly supports the pre-filtering and removal of all human reads prior to microbial metagenomic profiling.

We then tested HAYSTAC's performance when profiling samples with damage patterns characteristic of aDNA (*i.e.*, sequence fragmentation and cytosine deamination). To do so, we generated eight simulated samples consisting of 10 million reads each, both with and without aDNA damage (here-

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Table 1. Characteristics of the six simulated samples sets of the Simple, Random and Oral microbiome datasets.

Simulation set	Dataset	Number of replicates	aDNA damage	Mean number of species
Simple with human DNA	Simple Microbiome	2	No	10
Simple without human DNA	Simple Microbiome	2	No	10
Microbiome 100 species ancient	General Microbiome	2	Yes	100
Microbiome 100 species modern	General Microbiome	2	No	100
Microbiome 500 species ancient	General Microbiome	2	Yes	500
Microbiome 500 species modern	General Microbiome	2	No	500
Oral microbiome aDNA damage	Oral Microbiome	6	Yes	178
Oral microbiome modern	Oral Microbiome	6	No	178

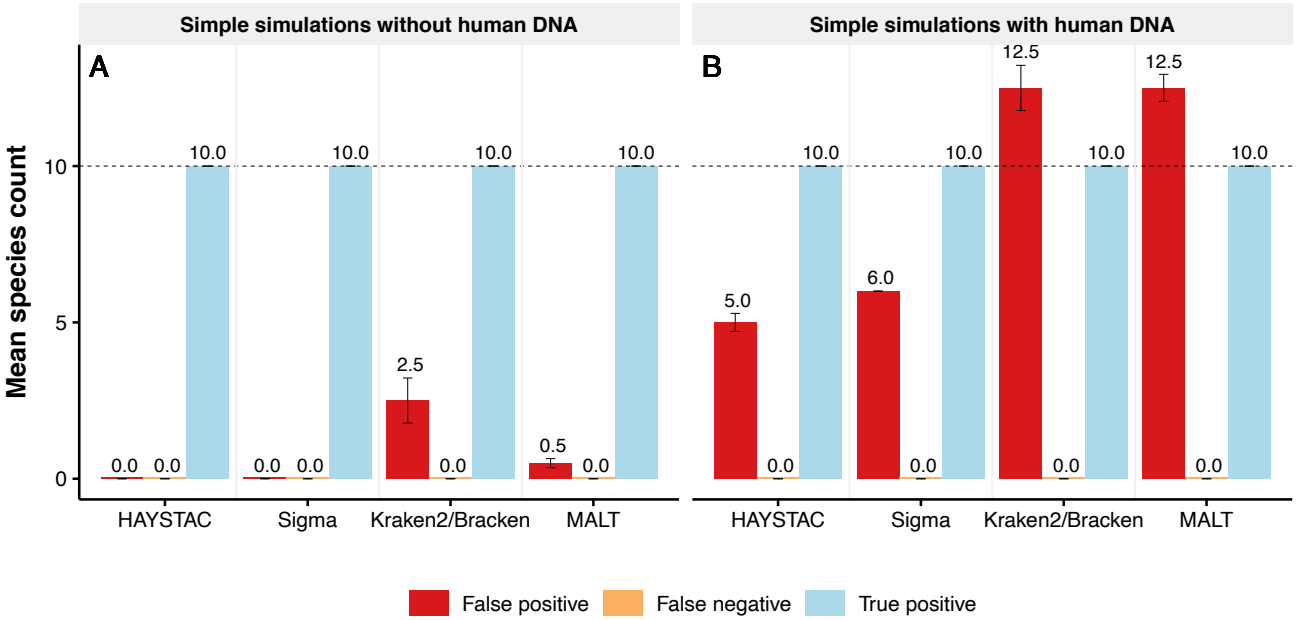

Figure 3. Accuracy of HAYSTAC and other methods for a simple simulation. Bar plot showing the mean count of false positives (red), false negatives (orange), and true detected species (blue) for two versions of the simple simulations dataset, each with two replicates: (A) without human DNA contamination ($n = 2$); and (B) with human DNA contamination ($n = 2$). The dotted line shows the number of simulated species in each set of samples (*i.e.*, the maximum true positive; $n = 10$), and numbers above the error bars indicate the mean species count in each category. For the simulation without human contamination, HAYSTAC outperforms both Kraken2/Bracken and MALT, and performs equally with Sigma (*i.e.*, no false positives or false negatives). For the simulation with human contamination, HAYSTAC outperforms all three other methods. All of the 5 false positive species identified by HAYSTAC are known to contain human sequences in their reference genomes (Breitwieser et al., 2019), confounding any analyses which do not explicitly filter human contamination.

after "General Microbiome" dataset). Each sample contained simulated reads from either 100 species (four simulated samples; two random sets of species all with equal abundance) or 500 species (four samples; two random sets of species all with equal abundance) that were randomly sampled from the RefSeq representative prokaryotic database (Table1).

Overall, the incidence of false negatives was low. HAYSTAC reported 2.0 false negative species on average in the 100 species sample set, and 14.0 in the 500 species sample set. Sigma reported 2.0 and 15.5, Kraken2/Bracken 2.75 and 23.5 and MALT 8.0 and 40.75, respectively (Supplemental Figure 2 and Supplemental Figure 3 A&B). False positives, in contrast, were variable between methods. HAYSTAC reported an average of only 0.5 false positives in the 100 species sample set, and an average of 1.0 false positives in the 500 species sample set. Sigma performed similarly, with an average of 0.5 and 1.0 false positives, while Kraken2/Bracken reported 11.5 and 17.0 false positives, and MALT reported 8.0 and 8.75 false positives in the 100 and 500 species sample sets, respectively (Supplemental Figure 3 A&B and Supplemental Table S3).

We next performed more realistic simulations based on species compositions that mimic an oral microbial community from a previously analysed subgingival plaque sample ((Velsko et al., 2018); hereafter "Oral Microbiome" dataset). These simulations included 12 simulated samples of 5 million paired-end reads each, with and without aDNA damage, and with 176–180 species in varying or constant species abundance (Table1). Out of the 196 species that were simulated for the Oral Microbiome dataset, only 115 were included in the RefSeq representative prokaryotic database. From these 115 species, only 4 species were simulated from the same genomes that were present in the modified representative prokaryotic RefSeq database we used (Supplemental

Table 2. False negative and false positive species counts in the ancient and modern Oral Microbiome datasets.

	Oral microbiome aDNA damage		Oral microbiome modern	
	False negative	**False positive**	**False negative**	**False positive**
HAYSTAC	73.2	29.7	73.2	35.3
Sigma	74.2	42.2	74.2	29.5
Kraken2/Bracken	74.0	170.7	73.8	224.2
MALT	75.0	137.2	74.8	157.5

Table S6).

As expected, given the incompleteness of the database, false negatives were elevated overall (Table 2). HAYSTAC failed to identify on average 73.2 species in the ancient (damaged) Oral Microbiome dataset, and 73.2 species in the modern Oral Microbiome dataset. Sigma failed to identify on average 74.2 and 74.2 species, Kraken2/Bracken failed to identify 74.0 and 73.8 species, and MALT failed to identify 75.0 and 74.8 species in the ancient and modern Oral Microbiome datasets, respectively (Figure 4 A&B and Supplemental Figure 4). False positives, however, were highly variable between methods. HAYSTAC reported an average of 29.7 false positives in the ancient Oral Microbiome dataset and 35.3 false positives in the modern Oral Microbiome dataset. Sigma reported 42.2 and 29.5 false positives, Kraken2/Bracken reported 170.7 and 224.2 false positives, and MALT reported 137.2 and 157.5 false positives in the ancient and modern datasets, respectively (Figure 4 A&B and Supplemental Table S4). Overall HAYSTAC outperformed the other methods in the ancient Oral Microbiome sample set with the lowest number of false positive identifications, and the highest number of true positive identifications in both the ancient and modern Oral Microbiome datasets.

Analyses of simulated data sets using genus-level database

We next assessed how accurately HAYSTAC identified specific candidate species using smaller reference databases. To do so, we re-analysed 20 simulated samples included in the General and Oral Microbiome datasets (General Microbiome 100 species, General Microbiome 500 species, Oral Microbiome aDNA damage, and Oral Microbiome modern datasets) (Table1) using nine different genus-specific databases (see Methods). For each randomly selected genus, we constructed a database that included all genomes found in the RefSeq representative prokaryotic database. The nine genera that were selected for this analysis were: *Bacteroides*, *Burkholderia*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Desulfitobacterium*, *Mycobacterium*, *Solimonas*, and *Streptococcus*. Each database consisted of all RefSeq genomes from all species belonging to the genus instead of the full RefSeq representative prokaryotic database. This resulted in databases with between 3–136 species each, as opposed to $5,636$ species when using the full RefSeq representative prokaryotic database. For each analysis, we computed false positive, false negative, and true positive species counts as before.

Overall, false negatives were low. HAYSTAC reported on average 0.1 species missing in the samples of the General and Oral Microbiome datasets, while Sigma reported 0.1, Kraken2/Bracken 0.1 and MALT 0.1 species missing (Figure 5, Supplemental Table S5). False positives, however, were variable between methods. HAYSTAC reported an average of 0.8 false positives, averaged across all samples in the General and Oral Microbiome datasets, while Sigma reported 1.1, Kraken2/Bracken 33.1, and MALT 29.1 (Figure 5). We also used HAYSTAC to compare the mean posterior abundance computed using the full RefSeq database with abundance computed using the genus-specific databases (Figure 6). We found that while genus-specific abundances were in cases slightly higher than those computed with the full RefSeq database, there was a very strong correlation between the two ($R^2 = 0.999$; slope$= 1$; p-value$= 0$; GLM: $y = x + 3.1 \cdot 10^{-6}$).

Pathogen identification from metagenomic communities

We then tested the ability of HAYSTAC and HOPS (a extension of MALT) to distinguish specific human pathogen species from close relative when occurring at low abundance in a metagenomic sample. To do so we selected 100 (human) pathobionts, each one belonging to a different genus, as well as 1 non-pathogenic microbial taxon from each of the selected genera (total of 200 taxa, 2 per genus, Supplemental table S11). We subsequently simulated a total of 200 libraries (1 library per species), each containing 100 reads with damage patterns characteristic to aDNA. This data data was then added to one of our previously simulated Oral Microbiome dataset samples. This resulted in 200 replicates of an Oral Microbiome sample (anc200e2repgn), with each replicate containing 1 human pathobiont at low abundance. Due to the the lack of taxonomic specificity present in a random sample of only 100 reads per pathogen genome, the overall true positive rate for this test was low. HAYSTAC identified 2016 true positive pathogenic reads, and misidentified 50 reads across the 200 replicates while HOPS only identified 144 true positive pathogen reads, and misidentified 58 reads across all replicates (Figure 7).

Case study 1: Ancient *Yersinia pestis*

We then tested HAYSTAC's performance to detect specific pathogens in real archaeological samples. We first analysed 130 publicly available plague-positive ancient human libraries (Rasmussen et al., 2015), obtained from seven bone and tooth samples (RISE00, RISE139, RISE386, RISE397, RISE505, RISE509 and RISE511).

HAYSTAC was run using a reference database consisting of the longest complete genomes for each of the species in the genus *Yersinia* (including plasmid sequences). Our method was able to confidently assign between $6,720$ and

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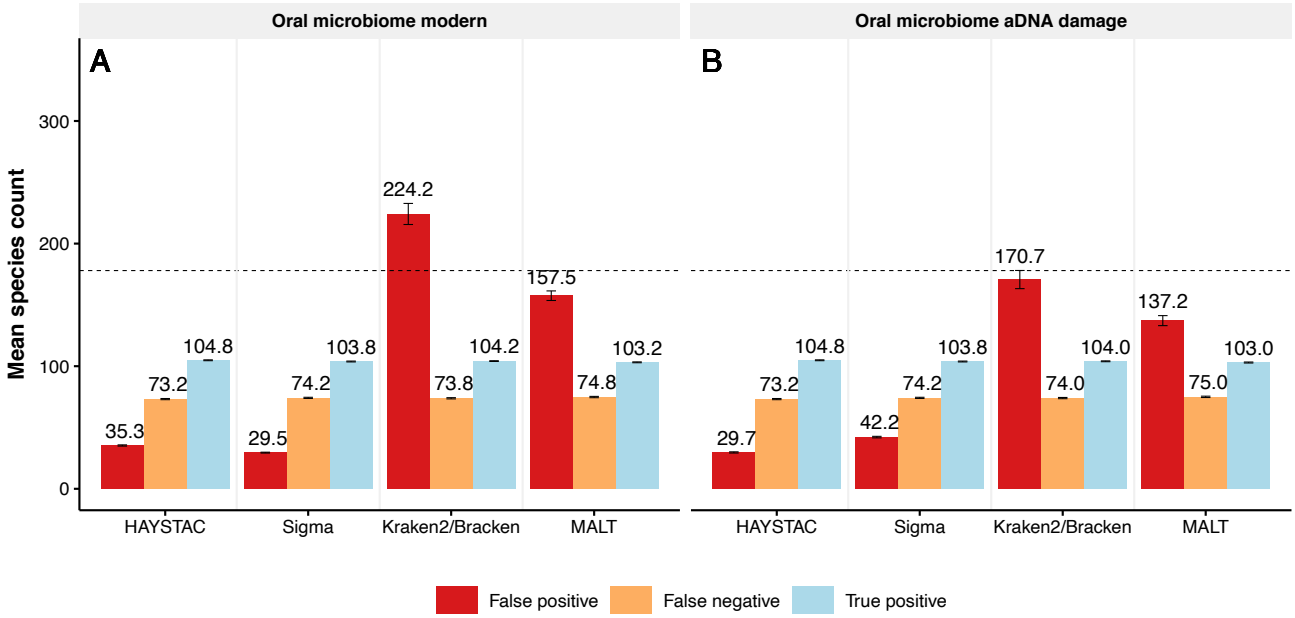

Figure 4. Accuracy of HAYSTAC and other methods for an oral microbiome simulation. Bar plot showing the mean count of false positives (red), false negatives (orange), and true detected species (blue) for two versions of the oral microbiome dataset, each with six replicates: (A) modern simulation, with fixed read lengths ($n = 6$); and (B) ancient simulation, with variable read lengths and post-mortem damage ($n = 6$). The dotted line shows the average number of simulated species in each set of samples (*i.e.*, the maximum true positive; $n = 178$), and numbers above the error bars indicate the mean species count in each category. For the modern simulation, HAYSTAC substantially outperforms both Kraken2/Bracken and MALT with respect to false positives, and performs equivalently with Sigma. For the ancient simulation, HAYSTAC outperforms all three other methods with respect to false positives. The overall high rates of false negative identifications are due to the absence of many simulated species from the reference database for all four methods. HAYSTAC also outperforms all other 3 methods in both the modern and ancient Oral Microbiome datasets by identifying the highest number of true positive species.

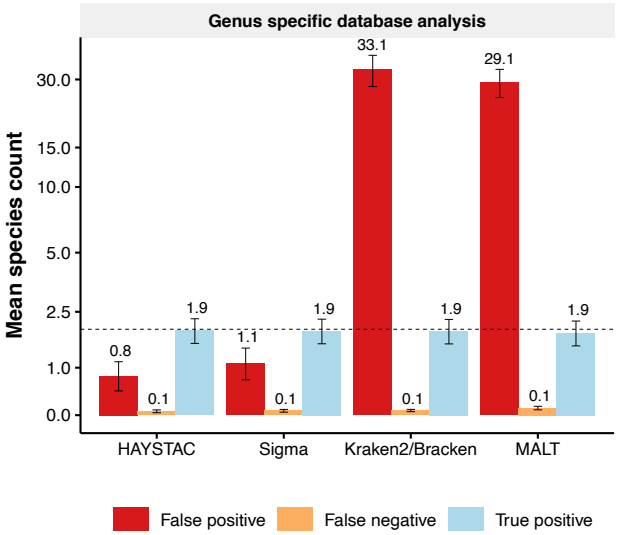

Figure 5. Accuracy of HAYSTAC and other methods using a reference database restricted to a single genus. Bar plot, with a pseudo-log10 transformed y-axis, showing the mean count of false positives (red), false negatives (orange), and true detected species (blue) for nine different genera (*Bacteroides*, *Burkholderia*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Desulfitobacterium*, *Mycobacterium*, *Solimonas* and *Streptococcus*), each with 20 samples from the general and oral microbiome datasets. The dotted line shows the average number of simulated species in each set of samples (*i.e.*, the maximum true positive; $n = 1.9$), and numbers above the error bars indicate the mean species count in each category. For the genus specific analysis, HAYSTAC substantially outperforms both Kraken2/Bracken and MALT with respect to false positives, and performs even better than Sigma.

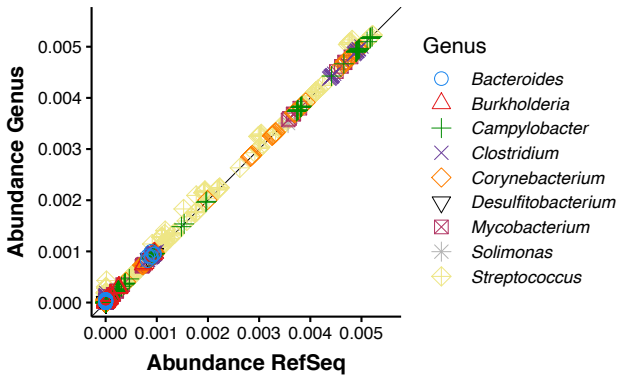

Figure 6. HAYSTAC inferred posterior abundance levels. Scatter plot showing the mean posterior abundances across all taxa ($n = 355$) and samples ($n = 20$) for either a genus specific database or the entire RefSeq representative database of prokaryotic species. Using a genus specific database has a small positive bias in mean posterior abundance for taxa within that genus (paired t-test p-value $< 2.2 \cdot 10^{-16}$, mean of the differences = $3.9 \cdot 10^{-6}$), nevertheless the overall abundance levels are highly correlated ($R^2 = 0.999$). Computational runtime for the genus specific analyses are approximately 350 times faster and use 5 times less memory, making genus specific analyses suitable for rapid initial screening.

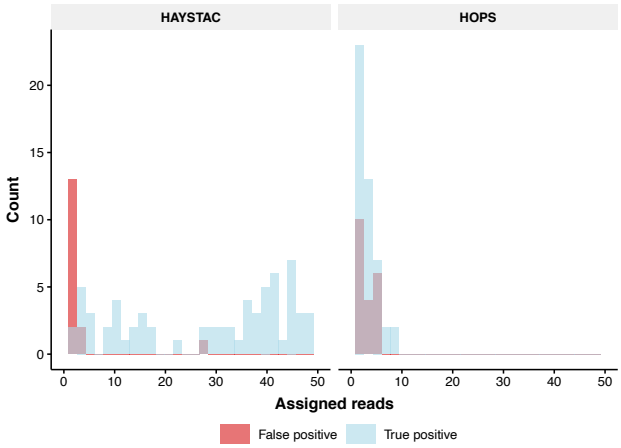

Figure 7. Histogram of the number of assigned pathobiont simulated reads. Histogram showing the the read count frequency of true positive (blue) and false positive (red) pathobiont reads as identified by HAYSTAC and HOPS, after screening 200 spiked iterations of an ancient Oral Microbiome dataset sample (anc200e2repgn). HAYSTAC identifies robustly more pathobiont reads than HOPS, while producing less false positive identifications.

856,467 reads to *Y. pestis* across all seven samples (Figure 8). HAYSTAC on average uniquely assigned an order of magnitude fewer reads compared to the number of aligned reads reported in the original study (Rasmussen et al., 2015). In addition, we found evidence for additional *Yersinia* species in all samples, including *Y. pseudotuberculosis* (606–72,581 reads), *Y. similis* (376–26,010 reads), *Y. canariae* (74–8,868 reads), *Y. enterocolitica* (209–5,040 reads), *Y. mollaretii* (147–24,241 reads) and *Y. ruckeri* (211–4,670 reads). Of these, *Y. ruckeri* and *Y. similis* are known to cause infections in animals (specifically fish), but have also been reported to infect humans (De Keukeleire et al., 2014).These identifications, however, are likely surprious, since *Y. ruckeri* and *Y. similis* are phylogenetically basal to other *Yersinia* species (Reuter et al., 2014; Chen et al., 2010) and may be attracting reads belonging diverged sequences.

The posterior abundances for *Yersinia pestis* in the RISE samples ranged from 0.0093–1.02%, corresponding to between 6,720–856,467 assigned reads. Despite the large number of assigned reads, posterior abundances only exceeded 0.01% for six of the seven samples (RISE00, RISE139, RISE386, RISE397, RISE505 and RISE509). This highlights the need to consider both relative and absolute abundance when making positive identifications.

Case study 2: Historic dental calculus

Dental plaque is a biofilm that develops naturally on teeth, and during life it periodically mineralizes to form dental calculus (Warinner et al., 2015), a robust substrate that can preserve biomolecules such as DNA from the biofilm and the host (Warinner et al., 2014). Although this substrate can act as a reservoir for respiratory pathobionts, analysis of ancient dental calculus metagenomic data is often challenging because the low abundance of these species makes it difficult to confidently identify them (Warinner et al., 2014).

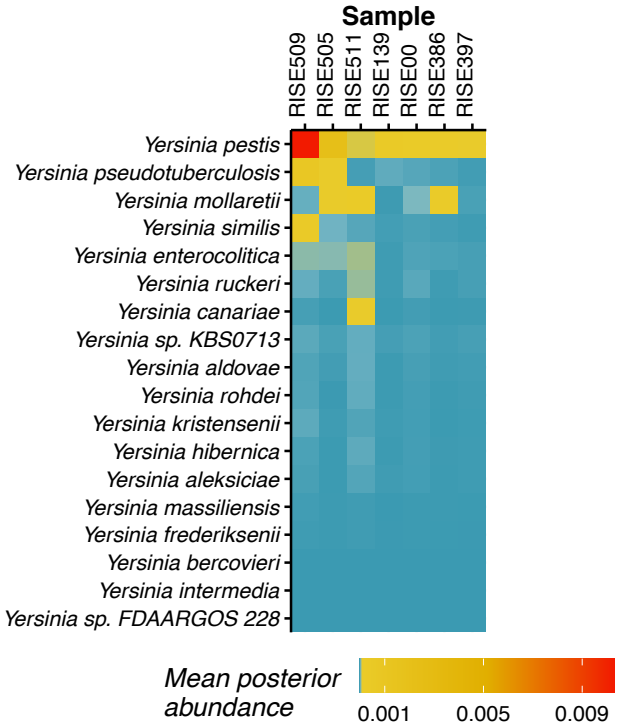

Figure 8. Posterior abundances of *Yersinia* species in Case Study 1. Heatmap showing the mean posterior abundances for the seven RISE samples, based on a genus specific analysis of 18 *Yersinia* species. *Yersinia pestis* is the species with the highest posterior abundance, followed by *Y. pseudotuberculosis*, in agreement with the results of (Rasmussen et al., 2015).

To test the performance of HAYSTAC on pathobiont aDNA sequences within host-associated ancient microbiomes, we analysed data from 48 dental calculus samples from a 19th century hospital in Oxford (Loe et al., 2014; Velsko et al., 2019).

We screened these libraries for potential upper respiratory pathobionts from the *Corynebacterium*, *Streptococcus*, *Klebsiella* and *Bordetella* genera (Figure 9), in an effort to test if such pathobionts could be preserved and identified in dental calculus. Members of the genera *Corynebacterium*, *Streptococcus* and *Bordetella* had also been included in the Oral Microbiome dataset. Within the *Corynebacterium* genus, we identified *Corynebacterium matruchotii* (abundance 0.01–1.96%; 37 samples) and *C. durum* (abundance 0.01–0.59%; 15 samples) both of which are oral commensal taxa. We did not, however, identify *Corynebacterium diphtheriae*, the species that causes diphtheria. We identified multiple species of *Streptococcus*, with the commensal *Streptococcus sanguinis* being identified in 33 samples at relatively high abundance (0.01–2.62%). The pathobiont *Streptococcus pneumoniae* was also positively identified at low abundance (0.01–0.07%) in 10 samples. We did not find any evidence for species belonging to the genera *Klebsiella* and *Bordetella*.

Members of the *Haemophilus* genus were rarely identified, which is expected given that many species from that genus are generally low-abundance in dental plaque compared to

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other oral surfaces such as the tongue (Mark Welch et al., 2016), and are similarly low-abundance in dental calculus (Velsko et al., 2019). However, *Haemophilus parainfluenzae* was found in 18 samples (abundance between 0.01–0.46%), *H. haemolyticus* was identified in two samples (abundance 0.01–0.03%), and *H. parahaemolyticus* was identified only in sample CS18 (abundance 0.02%). An uncharacterized oral species, *Haemophilus oral taxon 036*, was identified in three samples (abundance 0.01–0.04%) and the commensal *H. sputorum* was also identified in three samples (abundance 0.01–0.03%).

As expected, our analysis indicates that while species naturally occurring in the oral cavity (e.g. *C. matruchotii* and *durum)*, were detected in most samples, upper respiratory system pathobionts (e.g. *S. pneumoniae*, *H. parainfluenzae*) were more rare. Although pathogens were found generally at very low abundance, in only a few individuals, our results indicate that HAYSTAC was able to positively identify some potentially pathogenic species such as *Streptococcus pneumoniae* and *Haemophilus parainfluenzae*, suggesting that dental calculus is a promising substrate to study ancient respiratory pathobionts.

Discussion

Our results demonstrate that HAYSTAC provides a robust and computationally efficient framework to statistically assess the presence of specific species in a metagenomic sample. HAYSTAC identified fewer false positives than either Kraken2/Braken or MALT in all simulations, and fewer than Sigma in simulations of ancient data. It uses less memory than Kraken2/Braken and MALT. Furthermore, our results show that the use of reduced reference databases (e.g. a few selected genera as opposed to the entire RefSeq representative prokaryotic database), can dramatically reduce computational time (approximately 350 times faster) while still providing highly accurate species identification. Altogether, this makes HAYSTAC a flexible tool to rapidly and accurately profile metagenomic samples and screen them for pathogens, a feature that is attractive for a large range of applications, including archaeogenetics and biosurveillance.

Species concept, horizontal gene transfer and species identification

Although we demonstrated that both HAYSTAC and Sigma provide more robust species assignments than MALT or Kraken2/Bracken, their internal computation is based on the assumption that intraspecific polymorphism is lower than interspecific polymorphism. While this is true in most cases, the concept of a species among microorganisms is often not straightforward. This is the case, for example, in the *Mycobacterium tuberculosis* complex in which interspecific variability is very low (Tortoli, 2012), and occasionally lower than the default value of expected mismatch (*i.e.*, expected level of intraspecific polymorphism) set in HAYSTAC ($\sigma = 0.05$; see eq. 4 below). HAYSTAC deals with this issue by introducing an "ambiguous source" category, to which reads that are equally likely to match two or more genomes are assigned. This ensures that they do not contribute to posterior abundance computations, which could possibly force false identifications, while also not dismissing them into "unknown source" category with reads that cannot be classified. In cases where the levels of intra- and interspecific diversity are known (e.g., within a genus) it is possible to adjust the probability of the expected mismatches between a read and a genome (σ; see eq. 4), which can increase power while reducing false positive rates when analysing a specific dataset. This feature means HAYSTAC's performance can be fine-tuned to specific questions and datasets.

Identifying species within a metagenomic sample can also be complicated by horizontal gene transfer (HGT). HGT can lead to spurious, highly supported, species assignments—especially if the sequences originate from an outgroup species that is not represented in the database. HAYSTAC mitigates this issue by inspecting evenness of coverage across the genome for shallow sequencing data. Specifically, reads that belong to an outgroup species and that map to a genome in a reference database because of an HGT or due to contamination are expected to map only to parts of the reference genome (i.e. the part that was horizontally transferred). HAYSTAC assesses whether reads are clustered more than expected by chance across a reference genome using an evenness of coverage metric (see Methods). This provides the user with a quantification of the evenness of spread, and thus a proxy signature of potential HGT or misassignment of reads from a taxon that is not represented in the reference database.

Issues with databases

Another issue facing metagenomics is contaminated sequences incorporated into reference genomes. For example, multiple bacterial RefSeq representative assemblies contain human sequences (Breitwieser et al., 2019). This can lead to spurious, highly supported, species assignments when profiling metagenome samples. Including genome sequences from potential contaminant sources (e.g., the human reference genome) in the database should alleviate these issues (Feuerborn et al., 2020). Alternatively, it is also possible to filter reads before starting a taxonomic identification/assignment analysis (*i.e.*, by excluding those that map to the contaminant's reference genome). Potential contaminants, however, are not always known, and there are many gaps in reference sequence databases. In fact, inadequate species representation in databases is likely to be an important source of false positives for all species identification methods (Warinner et al., 2017).

Although reads belonging to a species that is not represented in a database are typically assigned to a higher taxonomic node ("ambiguous source" category in HAYSTAC), incorrect species assignment can occur if the missing taxon is closely related to another species in the database, or if the read derives from a conserved element found within a taxonomic

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Figure 9. Posterior abundances of oral microbiome species in Case Study 2. Heatmap showing the mean posterior abundances for the 44 dental calculus samples, based on a custom database that combined the prokaryotic representative RefSeq and pathobionts with complete genomes from the following 5 genera: *Corynebacterium*, *Haemophilus*, *Klebsiella*, *Streptococcus*, and *Bordetella*. Species from these five genera of interest that naturally colonise the oral cavity can be found in more samples and at higher abundance (e.g. *C. matruchotii*) compared to pathobionts of the upper respiratory system (e.g. *S. pneumoniae*).

group with sparse representation in the database. Although this is not considered a major problem for general profilers, as closely related species are likely to play a similar function in the ecosystem, such misassignments can greatly complicate the identification of specific pathogen species, especially if they have close environmental relatives.

Conclusions

Metagenomic data are now routinely produced and analysed, providing new insights into microbial communities that were previously unknown. Development of metagenomic analytical tools, however, has mostly focused on frameworks that can profile an entire microbial community. We showed that these methods, however, can produce high levels of false positives when focusing on specific species identification. Here we present a tool that can robustly assess whether a specific species is present in a metagenomic sample in one step, without the need to combine different pipelines to validate the results. HAYSTAC provides a user-friendly and automated method for rapidly constructing databases, and produces robust identifications regardless of the database size, allowing for both rapid hypothesis driven analyses and exhaustive profiling. Because of the mathematical framework it employs, HAYSTAC reliably produces the lowest number of false positive identifications, making it a valuable tool for both ancient and modern DNA microbial research.

Methods

Single Source Identification

We collect a series of observations (reads:$r_1 \cdot r_n$) and we denote the full set of observations as R. We map all the reads to a database of reference genomes and we define the event of sampling from the i-th reference genome in our database as G_j. Assuming that all reads comes from the same source (*i.e.*, a single species represented in the database), the events of sampling from a given genome are exhaustive and mutually exclusive, *i.e.*

$$G_1 \cdots G_n | G_i \cap G_j = \emptyset, \quad \forall i \neq j \quad \text{and} \quad \bigcup_i^n G_i = \Omega$$

From Bayes Theorem we have:

$$P(G_j|R) = \frac{P(R|G_j)P(G_j)}{P(R)} = \frac{P(R|G_j)P(G_j)}{\sum_k P(R|G_k)P(G_k)} \quad (1)$$

Given that each read is a different realization of sampling process (*i.e.*, an independent observation), we can express the

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likelihood as:

$$P(R|G_j) = \prod_i P(r_i|G_j) \tag{2}$$

Therefore, the posterior probability of sampling from a given genome G_j is:

$$P(G_j|R) = \frac{P(G_j)\prod_i P(r_i|G_j)}{\sum_k P(G_k)\prod_i P(r_i|G_k)} \tag{3}$$

Likelihood

We next derive a likelihood function to express the probability of a read given a genome $P(r_i|G_j)$. To do so, we use a previously published expression of this likelihood (Ahn et al., 2015) based on the number of mismatches between a read and a reference to which it is aligned:

$$P(r_i|G_j) = \sigma^z(1-\sigma)^{l-z}; \qquad z \leq U$$
$$P(r_i|G_j) = 0; \qquad z > U$$

where z is the number of mismatches between read r_i and genome G_j, U is the maximum number of mismatches allowed in the alignment and l is the length of read r_i (in bp).

The method assumes a uniform probability of mismatch within a given alignment (σ) to account for population level variability and sequencing errors. We expand this framework to leverage the fact that transitions and transversions occur at significantly different rates, particularly in ancient samples due to the effect of deaminations.

For each alignment we can then express the likelihood of generating a read r_i given that it has been sampled from the reference genome G_j based on both number of transitions (t) and transversions (v).

Let σ_t be the probability of observing a transition and σ_v the probability of observing a transversion, we can express the likelihood for a single read as:

$$P(r_i|G_j) = \sigma_v^{v_{i|j}}\sigma_t^{t_{i|j}}(1-\sigma_v-\sigma_t)^{l_i-v_{i|j}-t_{i|j}} \tag{4}$$

where l_i represent the length of the read r_i, while $v_{i|j}$ and $t_{i|j}$ denote the number of transversions and transitions between the read r_i and the genome G_j respectively.

Posterior

By substituting the likelihood term (eq. 4) into eq. 3, we can obtain an explicit form for the posterior probability of sampling a set of reads (R) from a given reference genome:

$$P(G_j|R) = \frac{\sigma_t^{\sum_i t_{i|j}}\sigma_v^{\sum_i v_{i|j}}(1-\sigma_v-\sigma_t)^{\sum_i(l_i-t_{i|j}-v_{i|j})}P(G_j)}{\sum_{k=1}^{k=n} P(G_k)\sigma_t^{\sum_i t_{i|k}}\sigma_v^{\sum_i v_{i|k}}(1-\sigma_v-\sigma_t)^{\sum_i(l_i-t_{i|k}-v_{i|k})}} \tag{5}$$

By rescaling both numerator and denominator by a factor of $(1-\sigma_v-\sigma_t)^{\sum_i l_i}$ we get:

$$P(G_j|R) = \frac{P(G_j)\left(\frac{\sigma_t}{1-\sigma_v-\sigma_t}\right)^{\sum_i t_{i|j}}\left(\frac{\sigma_v}{1-\sigma_v-\sigma_t}\right)^{\sum_i v_{i|j}}}{\sum_{k=1}^{k=n} P(G_k)\left(\frac{\sigma_t}{1-\sigma_v-\sigma_t}\right)^{\sum_i t_{i|k}}\left(\frac{\sigma_v}{1-\sigma_v-\sigma_t}\right)^{\sum_i v_{i|k}}} \tag{6}$$

Let $V_j = \sum_i v_{i|j}$ denote the total number of observed transversions between all reads and genome G_j while $T_j = \sum_i t_{i|j}$ represents the total number of transitions. Let $\delta_v = \frac{\sigma_v}{1-\sigma_v-\sigma_t}$ and $\delta_t = \frac{\sigma_t}{1-\sigma_v-\sigma_t}$ denote instead the transversion to match ratio and the transition to match ratio, respectively. By introducing this more compact notation, the previous equation can be re-written as:

$$P(G_j|R) = \frac{P(G_j)\delta_t^{T_j}\delta_v^{V_j}}{\sum_k^n P(G_k)\delta_t^{T_k}\delta_v^{V_k}} \tag{7}$$

Numerical Representation

Our method assumes a fixed probability of mismatches ($\sigma_v + \sigma_t$) which can be specified by the user and has a default value of 5%. We estimate the transition/transversion ratio based on the total number of mismatches per categories observed in the entire dataset and we obtain the value for σ_v and σ_t by solving the following linear system:

$$\begin{cases} \sigma_t + \sigma_v = 0.05 \\ \sigma_t = \frac{T}{V}\sigma_v \end{cases} \tag{8}$$

where $V = \sum_j\sum_i v_{i|j} = \sum_j V_j$ and $T = \sum_j\sum_i t_{i|j} = \sum_j T_j$.

If we assume a uniform prior in which each genome has a probability of $1/n$ we can simplify eq. 7 even further obtaining:

$$P(G_j|R) = \frac{\delta_t^{T_j}\delta_v^{V_j}}{\sum_k^n \delta_t^{T_k}\delta_v^{V_k}} \tag{9}$$

When analysing a large number of reads, evaluating eq. 9 might become problematic due to numerical representation issues. To accommodate for this we use an equivalent expression of the posterior probability:

$$P(G_j|R) = \frac{1}{1+\sum_{k\neq j}^n \delta_t^{T_k-T_j}\delta_v^{V_k-V_j}} \tag{10}$$

Each term in the summation at the denominator of eq. 10 represents the likelihood of genome G_k relative to genome G_J. There might be cases in which even the relative likelihood is so close to zero that cannot be represented numerically. We then take a conservative approach and assign to $\delta_t^{T_k-T_j}\delta_v^{V_k-V_j}$ the smallest number that the machine can represent (for a 64bit system it should be $2.2250738585072014 \cdot 10^{-308}$ which we roughly approximate with 10^{-300}).

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Multiple Sources Identification

We then expand this method to identify multiple species from metagenomic data. The multiple source identification approach involves the following steps:

- Assigning each read to a reference genome
- Obtain the likelihood for the multinomial distribution of abundances
- Obtain the posterior distribution from the Dirichlet distribution
- Calculate the 95% credible interval around each posterior mean
- Assess significance

Operatively we apply eq.9 to each read separately and obtain a posterior probability for a read to originate from a given genome:

$$P(G_j|r_i) = \frac{\delta_t^{t_{i|j}} \delta_v^{v_{i|j}}}{\sum_k^n \delta_t^{t_{i|k}} \delta_v^{v_{i|k}}} \tag{11}$$

Thus, for each aligned read we obtain a vector of posterior probabilities (one value for each genome in our dataset). If a posterior probability value is above a given threshold (default: 0.75) we consider the read r_i to be informative and assign it to that reference genome. If instead all posterior values are below the threshold, we consider the read to be uninformative and we assign it to the $n+1$ category where n is the number of reference genomes. This means that any read r_i that has been successfully aligned, can only be assigned to a single category: either to one reference genome or to the "Ambiguous Source" (AS, the $n+1$ category). All reads which belong to species that do not possess a representative reference genome (or a close relative) in the database and therefore haven't been successfully aligned to any of the reference genomes, will then be assigned to the $n+2$ category called "Unknown Source" (US).

Repeating this procedure $\forall r_i \in R$ allows us to populate a matrix $M_{N\times(n+2)}$ in which each row represents a read and each column represents a reference genome in our database or either the AS or US categories. Hence, the assignment matrix represents a series of N observations from a multinomial distribution of $n+2$ variables (where N is the total number of reads). This distribution is parametrized by a probability mass function $q = [q_1, q_2, ..., q_n, q_{AS}, q_{US}]$.

The likelihood of N observations has thus the following form:

$$\begin{aligned} &M \sim Multinomial_{n+2}(N, q) \\ &f(x_1, x_2, \cdots, x_{n+2}|N, q) = \frac{N!}{x_1! x_2! \cdots x_{n+2}!} \prod_{j=1}^{n+2} q_j^{x_j} \end{aligned} \tag{12}$$

where x_j is the sum of the column j of M, *i.e.* the number of occurrences of genome G_j.

Dirichlet prior

The Dirichlet distribution (Dir) is a conjugate prior for the multinomial distribution and it has desirable mathematical properties which make it appropriate to model the composition of metagenomes.

Let π_0 indicate the prior distribution and π^* the posterior.

From Bayes theorem,

$$\pi^*(M|\Theta) \propto L(M|\Theta)\pi_0(\Theta) \tag{13}$$

We employ a uniform prior:

$$\begin{aligned} &\pi_0(\Theta) \sim Dir(\alpha) \\ &\alpha_0 = (\alpha_1, \cdots, \alpha_{n+2}), \\ &\alpha_i = 1 \quad \forall i = 1, \cdots, n+2 \end{aligned} \tag{14}$$

Because the Dirichlet is a conjugate prior for the multinomial likelihood (eq. 12), the posterior density has the same form, *i.e*

$$\begin{aligned} &\pi_*(M|\Theta) \sim Dir(\alpha^*) \\ &\alpha^* = (\alpha_1^*, \cdots, \alpha_{n+2}^*), \\ &\alpha_j^* = \alpha_j + x_j,\ j = 1, \cdots, n+2 \end{aligned} \tag{15}$$

where, again, x_j is the number of observations (reads) of genome G_j in the assignment matrix M. We can then use this framework to compute the minimum abundance of a species (represented by its closest reference genome) in a metagenome.

Posterior mean and Confidence interval

Let γ_j denote the posterior abundance of genome G_j. From the property of the Dirichlet we can obtain the posterior mean as follows:

$$\begin{aligned} &E[\gamma_j|X] = \frac{\alpha_j^*}{\alpha^{**}} \\ &\alpha^{**} = \sum_{k=1}^{n+2} \alpha_k^* = (n+2) + \sum_{k=1}^{n+2} x_k = n+2+N \end{aligned} \tag{16}$$

meaning that the denominator of each posterior mean corresponds to the total number of reads plus the number of categories (the number of reference genome + 2).

The marginal distribution of each abundance is:

$$\gamma_j \sim Beta(\alpha_j^*, \alpha^{**} - \alpha_j^*) \tag{17}$$

and we can use this to obtain the 95% CI numerically.

Computational architecture

HAYSTAC is written in Python and built upon the snakemake workflow engine (Köster and Rahmann, 2012). It operates as a wrapper around custom python scripts and several common bioinformatics tools, including: AdapterRemoval (Schubert et al., 2016), bowtie2 (Langmead and Salzberg, 2012), DeDup (Peltzer et al., 2016), mapDamage (Jónsson et al., 2013), samtools (Li et al., 2009), SeqKit (Shen et al., 2016), seqtk (Li, 2012) and sra-tools (Leinonen et al., 2011). It uses the conda package manager, with bioconda (Grüning et al., 2018), to manage its dependencies.

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A tool for constructing databases

HAYSTAC includes a user-friendly tool to download genomes from NCBI and build customisable reference databases. After deciding *a priori* which taxa to include in their database (e.g., anywhere between a single genus to many thousands of taxa), the user constructs a database from any combination of: (i) an NCBI search query; (ii) a user specified list of NCBI accession codes; (iii) the RefSeq representative database of prokaryotic species; and/or (iv) a list of user provided reference assemblies (in FASTA format).

To construct a valid NCBI search query, visit the NCBI Nucleotide database website (https://www.ncbi.nlm.nih.gov/nucleotide/) and use the search feature to obtain a correctly formatted query string from the "Search details" box. This search query can then be used directly with HAYSTAC to automatically download and build a reference database based on the accession codes present in the resultset returned by the query.

We caution that the choice of reference database can dramatically affect species identification for all methods that use reference databases. For example, issues can arise if the reference genomes in the database are not complete, as reads may map uniquely to one reference genome because other incomplete genomes in the database do not possess that specific sequence. To mitigate this issue, HAYSTAC uses a simple heuristic to select the longest genome available per species in the input resultset, which limits issues arising from unequal size of reference genomes in the database.

Processing and aligning reads

Pre-processed reads can be directly passed onto the alignment step (Figure 1). This is useful when dealing with modern DNA. The user can also decide whether to remove sequencing adapters in the case of raw reads, and for ancient/degraded DNA collapse overlapping mate pairs using a wrapper for AdapterRemoval2 (Schubert et al., 2016). Reads are then aligned to a database using Bowtie2 (Langmead and Salzberg, 2012). We first align to a single Bowtie2 index that contains all the reference genomes, to obtain a BAM file that contains one single best alignment per read. For this alignment step we use the local alignment mode in Bowtie2. This allows us to discard reads that did not map to a single reference genome. This BAM is used by HAYSTAC to compute the expected T_s/T_v ratio from the data, which is used as prior in eq 4. Reads that pass this first filtering step are then re-aligned to each reference genome separately using Bowtie2 (one index per reference) in end-to-end mode. We set a maximum number of mismatches to calculate the minimum alignment score during the Bowtie alignment. The maximum mismatch allowed during this second alignment step is calculated by the average read length of the input library. Allowing more mismatches results in slower run time as the number of reads included in the analytical step increases.

Computing abundance

Alignment files are then processed to calculate a likelihood score for each read/reference alignment and build a likelihood matrix that possesses $n \times r$ entries, where n is the number of reference genomes and r is the number of reads that pass the first filtering stage. This is done by computing T_s, and T_v for each alignment, and computing the likelihood using eq 4. Here the user can change the probability of mismatch within a given alignment (σ; default 0.05) (see eq 4). For each read we then compute a posterior probability using eq 7. Reads are then filtered based on their posterior probability (default 0.75) and included in the Dirichlet assignment step. Reads that have not been aligned to any taxa in the database get assigned to the "Unknown Source" category, a category that absorbs reads whose reference genomes are not included in our database. Reads that have been aligned to multiple taxa in our database but obtain a posterior probability below the threshold that is set for the Dirichlet assignment are also assigned to another special category called the "Ambiguous Source", a category that includes reads that were not informative enough to be uniquely aligned to a specific taxon. Both of these categories are included in the mean posterior abundance calculations.

Assessing evenness of coverage

We use an evenness of coverage ratio to assess if the reads assigned to a specific taxon represent a random sample of its genome or whether they are clustering around specific regions. The evenness of coverage ratio is defined as the genome coverage of a taxon over the fraction of its genome that is covered by aligned reads. In the case of a true positive identification the reads should not be clustering around specific genome regions (evenness ration< 10 as estimated from empirical datasets), something that would indicate an HGT event. This test is particularly useful for shallow sequencing data that do not allow for more than $1\times$ genome coverage of a given reference genome.

Representative species RefSeq database

For our analyses we build a database out of all the representative species of the prokaryotic RefSeq database. A list of all the species that were included can be found here: https://ftp.ncbi.nlm.nih.gov/genomes/GENOME_REPORTS/prok_representative_genomes.txt. In cases where the RefSeq representative database contains more than one strain for a given species, the first listed accession was picked. We also added complete genomes from the genera *Klebsiella*, *Streptococcus*, *Corynebacterium*, *Bordetella*, *Haemophilus* and *Yersinia*, for these species that were not present in the prokaryotic representative RefSeq. When possible (*i.e.*, when genome assembly with plasmid data were available) we included plasmid sequences in the database.

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Simulations

We generated 3 different simulated datasets. The first dataset was generated to determine the accuracy of different methods under simple conditions. We did not apply any chemical damage pattern and the read length was kept constant at 60 bp. This was done by randomly sampling 10 species from the prokaryotic representative RefSeq database (see above) twice, to get two different sets of 10 species. For each set we then simulated 2 different sequencing library samples of 1 million single-end reads using gargammel (Renaud et al., 2017). One set had no human genome included and all taxa were present at 10% abundance. In the second set 25% of reads were from the human genome, and all remaining taxa were present at 7.5% abundance. This dataset included 4 simulated sample sequencing files.

The second dataset was produced to compare performance of the different tools when analysing ancient and modern DNA, and did not include the human genome. To do so, we first generated two sets of 100 species randomly sampled from the prokaryotic representative RefSeq database, and two sets of 500 randomly sampled species. The species were different between each of the 2 random samplings for both the 100 species and 500 species sets. For each species profile in both the 100 species and 500 species sets, we simulated two different libraries (8 total libraries) using gargammel with 10 million single-end reads of length 125bp from an Illumina HiSeq 2500 run. The species abundances were kept consistent within each library, where libraries of 100 species have all taxa at 1% abundance and libraries of 500 species have all taxa at 0.2% abundance. The first library incorporated aDNA damage patterns based on the profile of reads that mapped to the Tannerella forsythia genome from a real ancient dental calculus sample (CS21 from Velsko et al. (2019)) and the second library did not include aDNA damage patterns.

To assess our method with more realistic data sets, we analysed simulated data previously published by Velsko et al. (2018) designed to resemble a dental plaque community, which included species that were not in the prokaryotic representative RefSeq database. Twelve datasets of 200 species each were generated: with and without ancient DNA damage, each with even species abundance (0.5%) or with abundance based on values observed in an ancient oral microbiome bacterial community (see Supplemental Table S8). The type of simulation included members of the *Corynebacterium* genus without *Corynebacterium diphtheriae*; the second type included all members of the *Corynebacterium* genus (including *Corynebacterium diphtheriae*); the third type did not include any member of the *Corynebacterium* genus. Each of these twelve samples contained 5 million paired end reads of varying length, that were collapsed with AdapterRemoval.

Lastly, we simulated reads from human pathogens and closely related species to test HAYSTAC's ability to identify low abundance pathogens in metagenomic data. To do so, we simulated 200 single-end Illumina sequencing libraries from 100 pathobionts and 100 non-pathogenic closely related species repsectively, with aDNA damage patterns (estimated from simulated sample anc200e2repgn), which we then added to one of the simulated ancient Oral Microbiome dataset samples (anc200e2repgn). These libraries were then analysed using both HAYSTAC and HOPS (see below for more details).

Analyses of simulated data

We trimmed adapters using AdapterRemoval v2 (Schubert et al., 2016) excluding reads that were smaller than 15 bp. Analyses of simulated data with Kraken2/Bracken (Wood et al., 2019; Lu et al., 2017), MALT (Vågene et al., 2018) were conducted using default parameters. HAYSTAC and Sigma (Ahn et al., 2015) were run with default parameters. For HAYSTAC, Kraken2/Bracken and Sigma positive identification abundance threshold was set to 0.01% while we used 50 reads for MALT/HOPS as in Hübler et al. (2019).

For the pathogen detection analysis, HAYSTAC was run with a mismatch probability of 0.2 and a read assignment probability threshold of 0.5. For the HOPS analysis we initially ran cMALT (a version of MALT adapted to aDNA libraries) with the following flags activated $-asm-tails5$, while setting all the other parameters at default settings. The cMALT output was subsequently analysed with HOPS (Hübler et al., 2019) with default values but providing a list of candidate species that included 100 pathogen species, and using the outputs characterised as ancient by HOPS. Before counting the true and false positive reads assigned to pathogen taxa by each method, we removed the all the microbial taxa each method would identify in the initial library of the ancient Oral Microbiome sample anc200e2repgn, so that the background species composition of the library would not affect the identification of the spiked pathobionts.

In order to assess how HAYSTAC, Sigma, Kraken2/Bracken and MALT/HOPS perform with genus specific database, we analysed 20 simulated samples (based on RefSeq genomes; see above) and aligning reads to all representative genomes in a single genus. Ten genera were picked randomly for these analyses: *Bacteroides* (24 species), *Burkholderia* (3 species), *Campylobacter* (22 species), *Clostridium* (91 species), *Corynebacterium* (83 species), *Desulfitobacterium* (4 species), *Lactobacillus* (139 species), *Mycobacterium* (36 species), *Solimonas* (3 species) and *Streptococcus* (78 species).

Case study 1: Ancient *Yersinia pestis*

We tested HAYSTAC using a published dataset of ancient human bone and tooth samples in which *Yersinia pestis* was identified (Rasmussen et al., 2015), to compare performance against standard techniques used to identify specific pathogens in aDNA samples. We downloaded reads from all seven samples in which the authors detected *Y. pestis* (RISE00, RISE139, RISE386, RISE505, RISE509, RISE511) from the European Nucleotide Archive (NCBI BioProject accession: PRJEB10885). We then used

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HAYSTAC to build a database that contains the longest complete genome for each species of the genus *Yersinia* and aligned reads using default parameters. HAYSTAC was then used to compute posterior abundance of each species.

Case study 2: Historic dental calculus

We also tested HAYSTAC using an historic dental calculus dataset (Velsko et al., 2019) (PRJEB30331). Raw reads were processed as described in Velsko et al. (2019). We attempted to identify a set of respiratory pathogens in these calculus samples, including: *Corynebacterium diphtheriae* and *Bordetella pertussis*, the causative agents of diphtheria and whooping cough, respectively (Figure 8). We also tested for the presence of species belonging to the *Haemophilus* (ideally *Heamphilus influenzae* or *parainfluezae*), *Klebsiella* (ideally *Klebsiella pneumonae*) and *Streptococcus* (*Streptococcus pneumoniae*) genera. These species were selected to test if pathobionts of the upper respiratory system could be found in dental calculus.

Performance test

For the performance tests we created a reproducible conda environment, inside which all the different pipelines were installed and run. For each method we used the GNU time command to measure elapsed time (wall clock) and peak memory usage (maximum resident set size). Scripts to run the performance benchmarks are available from https://github.com/antonisdim/haystac_paper

We used a total of 500 accessions, one for each species, which were a subset of the accessions used for the analysis of all real and simulated datasets. We built databases with an increasing number of species (10, 100, 500) and the same database was given as input to all the methods. We only measured the database building time and maximum memory usage for each method, as HAYSTAC was used to efficiently fetch all 500 reference genomes from NCBI.

For sample inputs we used samples from the Human Microbiome Project, with accession SRS078671. We merged the forward mate and the singleton reads in one file that we then treated as single end reads. That file was subsequently subsampled to generate fastq files of 10 K, 100 K, and 1 M SE reads respectively, that were used as inputs for all the tests.

For the sample analysis we performed two tests, one that measured max memory usage and runtime for samples of increasing size against the database of 500 species, and a second where the input file size was kept constant at 1 million reads, while the database size was being varied, again measuring the same variables for memory and elapsed execution time.

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Competing interests

The authors declare no competing financial interests.

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HAYSTAC: Supplementary Information

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Calculation of false positive, false negative and true positive rates

For the calculation of the false positive, false negative and true positive rates we used the following formulas:

$$FP_r = \frac{FP}{FP+TN} \tag{1}$$

$$FN_r = \frac{FN}{FN+TP} \tag{2}$$

$$TP_r = \frac{TP}{TP+FP} \tag{3}$$

where: $TP =$ number of true positives, $FP =$ number of false positives, $TN =$ number of true negatives, and $FN =$ number of false negatives. For a true positive identification we require an abundance equal or higher than 0.01%.

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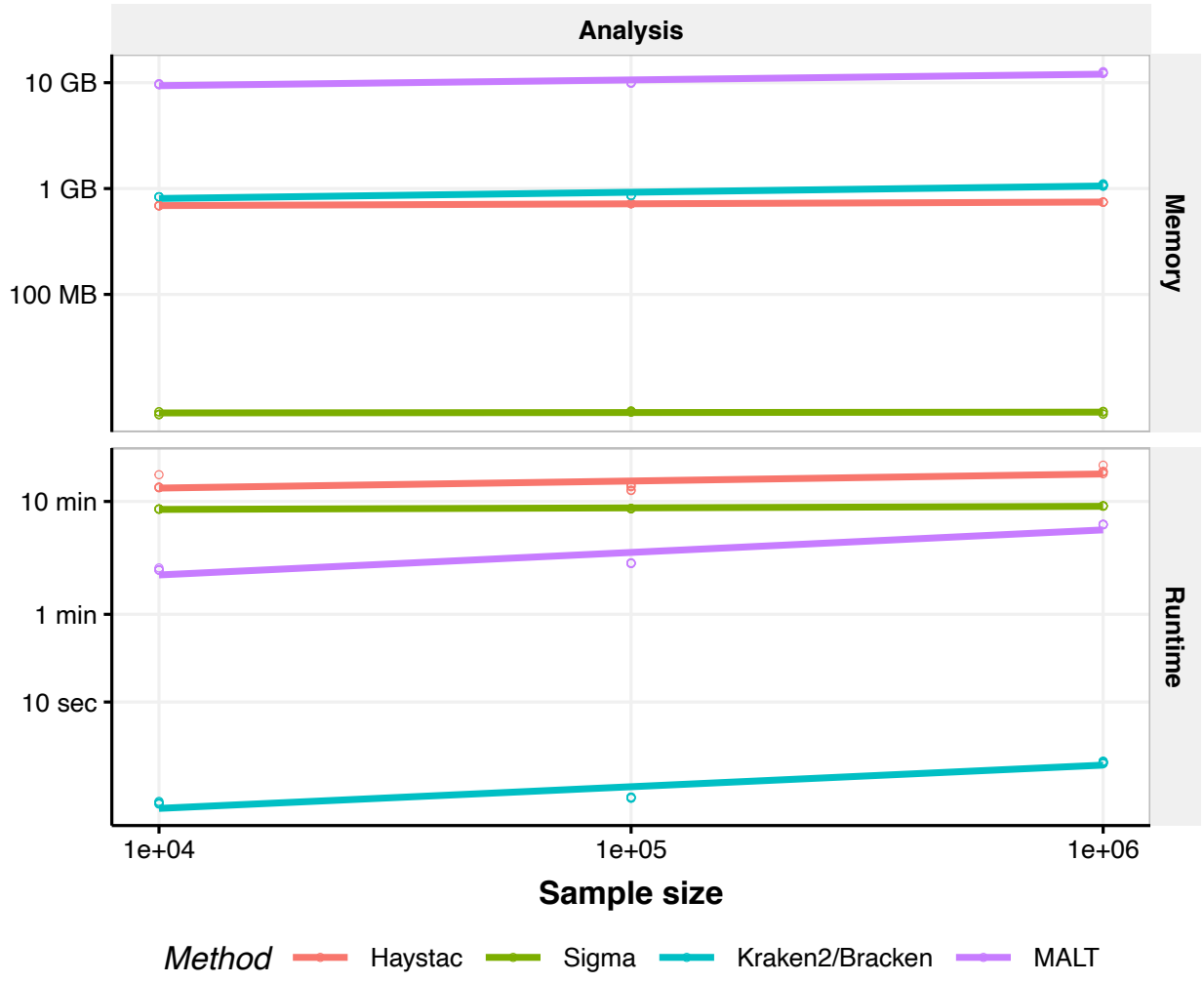
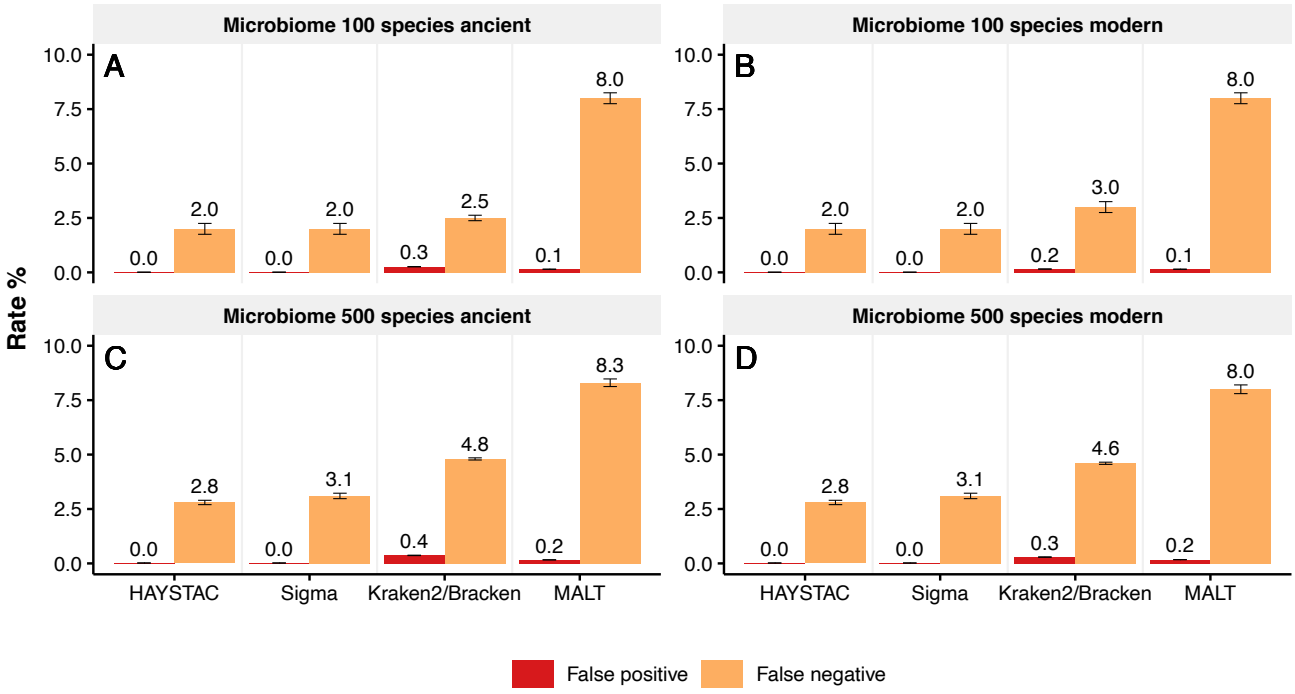
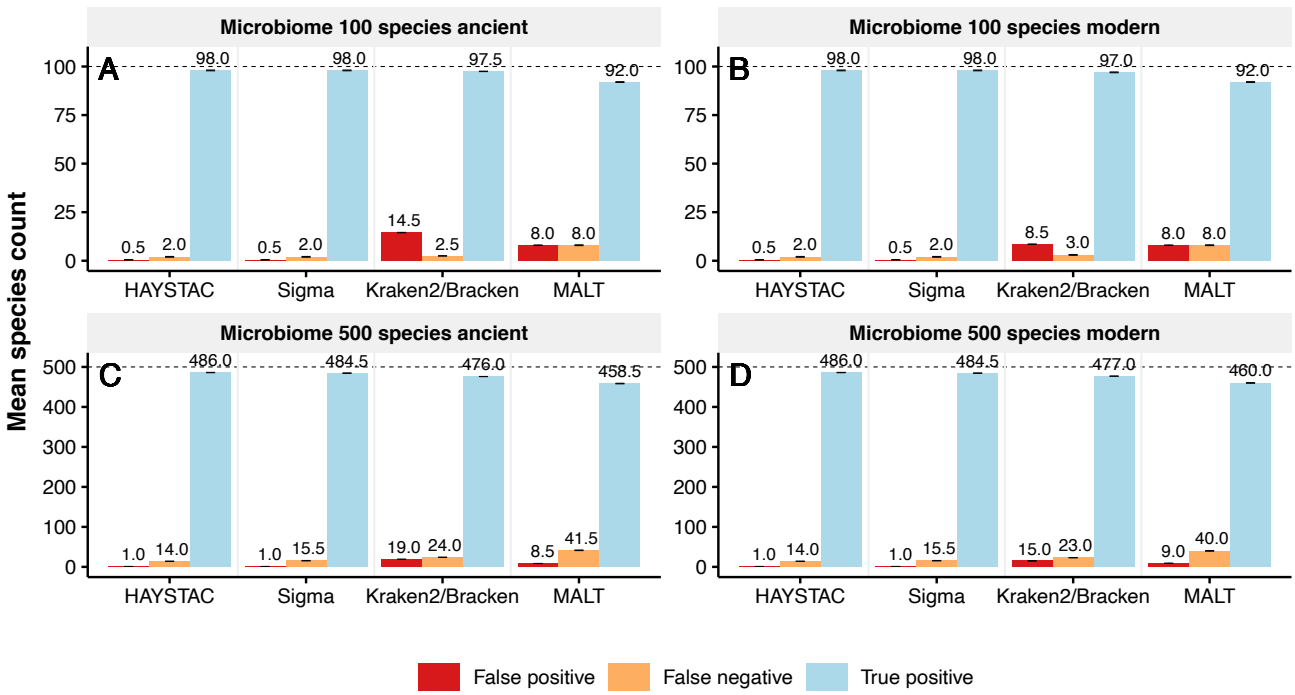


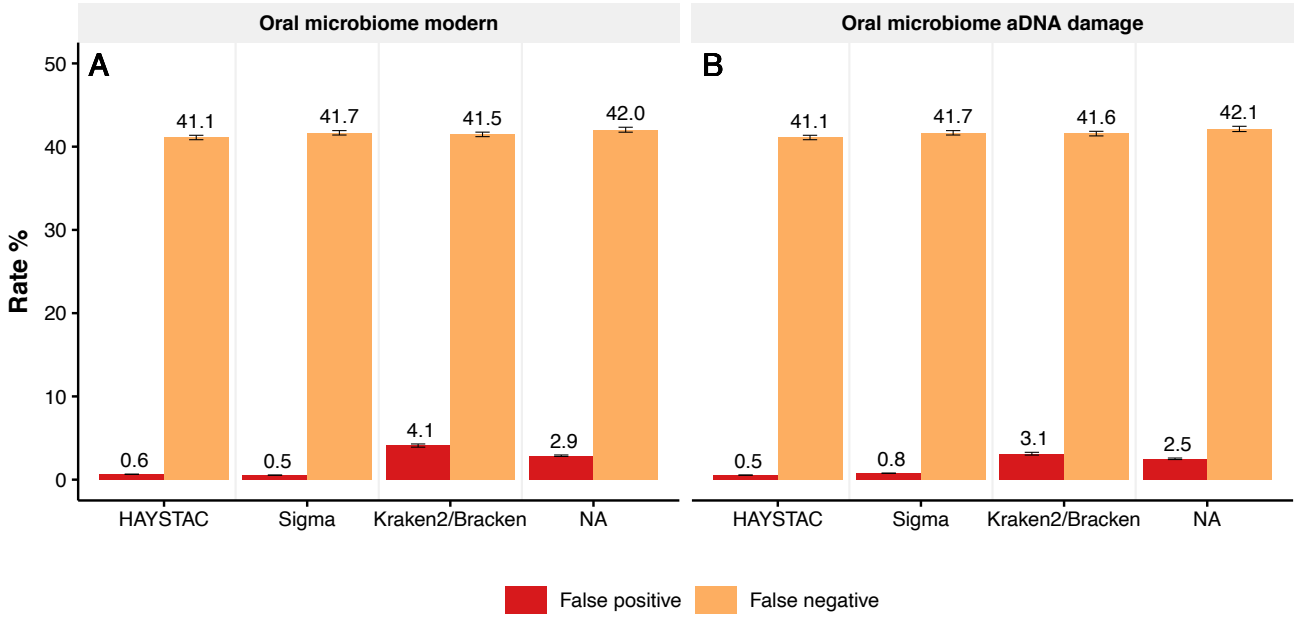
Supplemental Figure 1. Benchmarking for elapsed runtime and memory for HAYSTAC, Sigma, Kraken2/Bracken and MALT when analysing samples of 10 K, 100 K and 1 M reads against a database of 500 species. Memory remains constant as sample size increases, and runtime in most methods (other than Kraken2/Bracken) scales with the database size rather than input sample size).



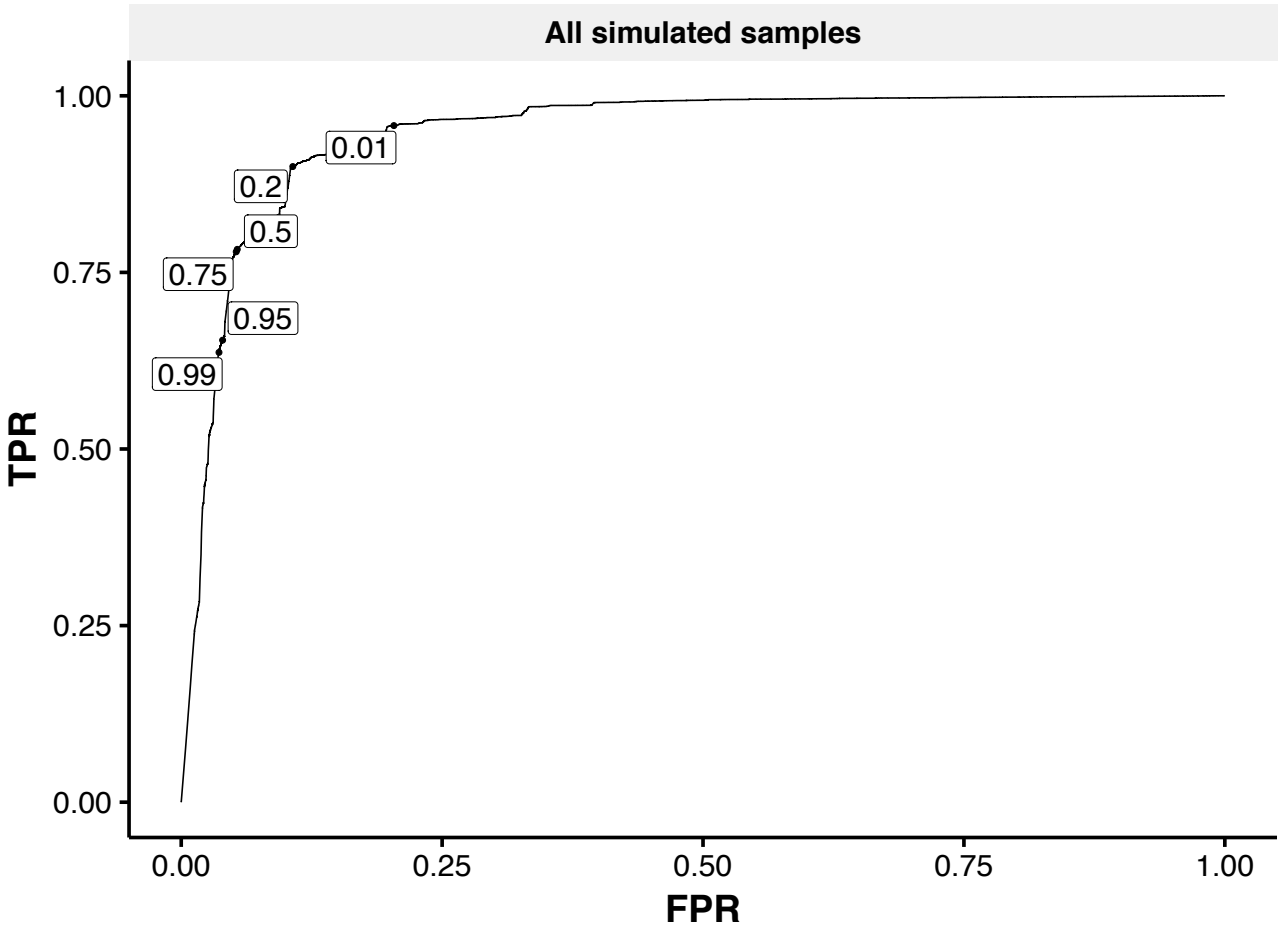
Supplemental Figure 2. False positive and negative rates per method for the simulated samples of the General Microbiome dataset.



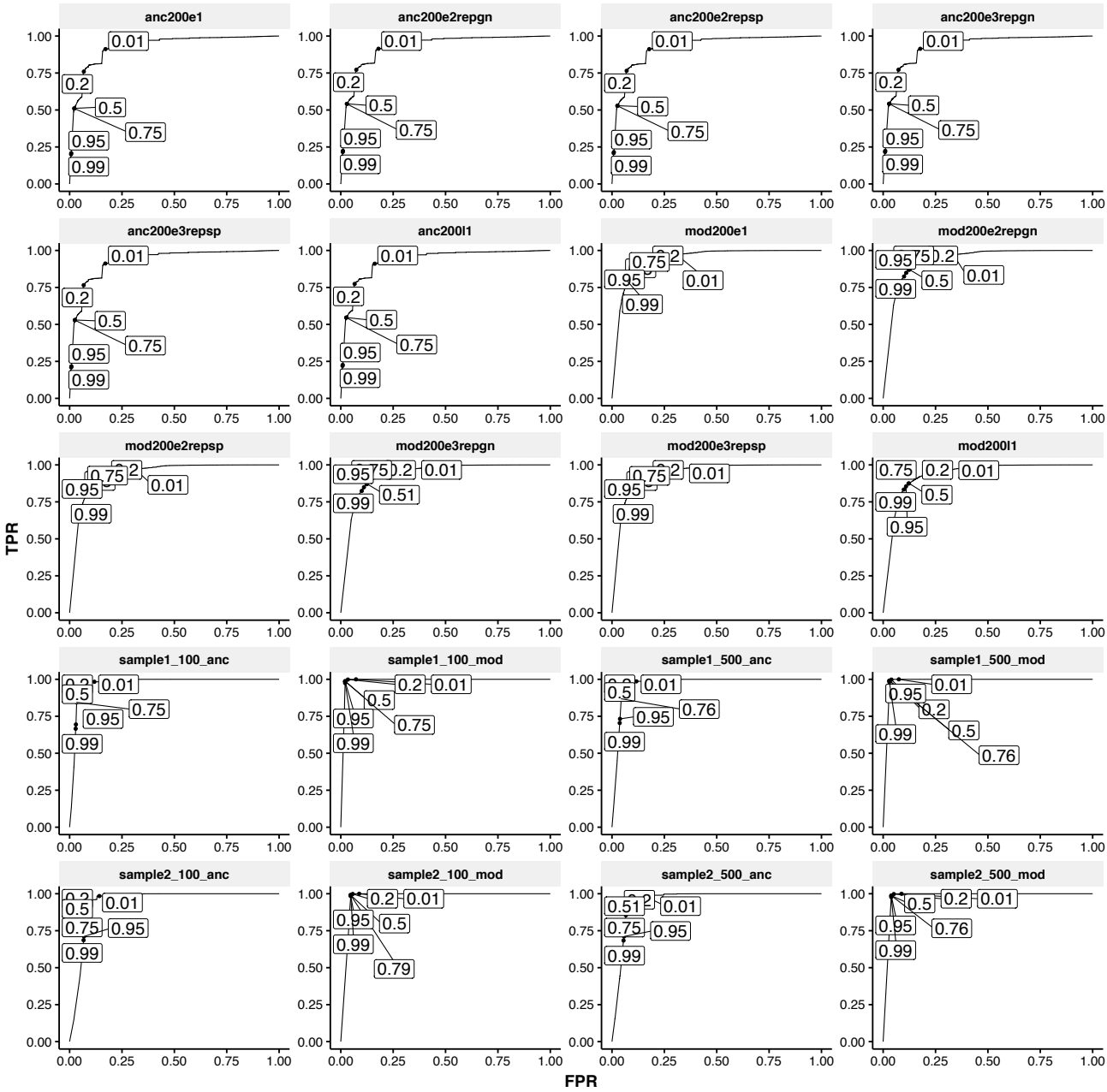
Supplemental Figure 3. Mean count of false positive (red), false negative (orange), and true detected species (blue) in the simulated General Microbiome dataset of 100 species ancient (n=2) (A), 100 species modern (n=2) (B), and 500 species ancient (n=2) (C) and 500 species modern (n=2) (D). The dotted line represents the average number of simulated species in each set of samples, and the numbers above the error bars represent the mean species count.



Supplemental Figure 4. False positive and negative rates per method for the simulated samples of the Oral Microbiome dataset.



Supplemental Figure 5. Receiver operator curve analysis, showing the relationship between the true and false positive ratios (TPR and FPR respectively) to determine the default read posterior probability threshold for the dirichlet assignment across all simulated samples. A rather conservative threshold of 0.75 is appropriate for most types of analysis. If the user requires a more relaxed threshold for discovery purposes the threshold can be lowered to 0.5. Respectively if a user is analysing deep sequencing data or wants to perform a more stringent identification the threshold can also be increased.



Supplemental Figure 6. Receiver operator curve analysis, showing the relationship between the true and false positive ratios (TPR and FPR respectively) to determine the default read posterior probability threshold for the dirichlet assignment per sample. Here we can see how the read length and deamination levels affect the true positive rate. The user might want to consider these additional factors if they wish to change the default value of 0.75.

Supplementary table S1. Characteristics of the General Microbiome simulated dataset.

Sample	Chemical damage pattern	Abundance	Read length	Number of species
sample1_100_anc	yes	1%	Variable	100
sample1_100_mod	no	1%	Variable	100
sample1_500_anc	yes	0.02%	Variable	500
sample1_500_mod	no	0.02%	Variable	500
sample2_100_anc	yes	1%	Variable	100
sample2_100_mod	no	1%	Variable	100
sample2_500_anc	yes	0.02%	Variable	500
sample2_500_mod	no	0.02%	Variable	500

Supplementary table S2. Characteristics of the Oral Microbiome simulated dataset.

Sample	Chemical damage pattern	Abundance	Read length	Number of species	*C. diphtheriae control*
anc200e1	yes	0.50%	80 bp	176	positive
anc200e2repgn	yes	0.50%	80 bp	180	negative - genus
anc200e2repsp	yes	0.50%	80 bp	178	negative - species
anc200e3repgn	yes	0.50%	80 bp	180	negative - genus
anc200e3repsp	yes	0.50%	80 bp	178	negative - species
anc200l1	yes	variable	80 bp	176	positive
mod200e1	no	0.50%	150 bp	176	positive
mod200e2repgn	no	0.50%	150 bp	180	negative - genus
mod200e2repsp	no	0.50%	150 bp	178	negative - species
mod200e3repgn	no	0.50%	150 bp	180	negative - genus
mod200e3repsp	no	0.50%	150 bp	178	negative - species
mod200l1	no	variable	150 bp	176	positive

Supplemental table S3. Species identification counts and rates for the General Microbiome simulated dataset.

Sample	Total Species	False Positive Count	True Detected Species Count	False Negative Count	False Positive Rate %	False Negative Rate %	Method
sample1_100_anc	100	0	97	3	0	3	haystac
sample1_100_mod	100	0	97	3	0	3	haystac
sample2_100_anc	100	1	99	1	0	1	haystac
sample2_100_mod	100	1	99	1	0	1	haystac
sample1_100_anc	100	15	97	3	0.3	3	kraken
sample1_100_mod	100	8	96	4	0.1	4	kraken
sample2_100_anc	100	14	98	2	0.3	2	kraken
sample2_100_mod	100	9	98	2	0.2	2	kraken
sample1_100_anc	100	8	93	7	0.1	7	malt
sample1_100_mod	100	8	93	7	0.1	7	malt
sample2_100_anc	100	8	91	9	0.1	9	malt
sample2_100_mod	100	8	91	9	0.1	9	malt
sample1_100_anc	100	0	97	3	0	3	sigma
sample1_100_mod	100	0	97	3	0	3	sigma
sample2_100_anc	100	1	99	1	0	1	sigma
sample2_100_mod	100	1	99	1	0	1	sigma
sample1_500_anc	500	1	484	16	0	3.2	haystac
sample1_500_mod	500	1	484	16	0	3.2	haystac
sample2_500_anc	500	1	488	12	0	2.4	haystac
sample2_500_mod	500	1	488	12	0	2.4	haystac
sample1_500_anc	500	17	475	25	0.3	5	kraken
sample1_500_mod	500	13	476	24	0.3	4.8	kraken
sample2_500_anc	500	21	477	23	0.4	4.6	kraken
sample2_500_mod	500	17	478	22	0.3	4.4	kraken
sample1_500_anc	500	8	455	45	0.2	9	malt
sample1_500_mod	500	9	456	44	0.2	8.8	malt
sample2_500_anc	500	9	462	38	0.2	7.6	malt
sample2_500_mod	500	9	464	36	0.2	7.2	malt
sample1_500_anc	500	1	482	18	0	3.6	sigma

sample1_500_mod	500	1	482	18	0	3.6	sigma
sample2_500_anc	500	1	487	13	0	2.6	sigma
sample2_500_mod	500	1	487	13	0	2.6	sigma

Supplemental table S4. Species identification counts and rates for the Oral Microbiome simulated dataset.

Sample	Total Species Count	False Positive Count	True Detected Species Count	False Negative Count	False Positive Rate %	False Negative Rate %	Method
anc200e1	176	30	104	72	0.5	40.9	haystac
anc200e2repgn	180	32	104	76	0.6	42.2	haystac
anc200e2repsp	178	28	107	71	0.5	39.9	haystac
anc200e3repgn	180	33	104	76	0.6	42.2	haystac
anc200e3repsp	178	27	107	71	0.5	39.9	haystac
anc200l1	176	28	103	73	0.5	41.5	haystac
mod200e1	176	36	104	72	0.7	40.9	haystac
mod200e2repgn	180	37	104	76	0.7	42.2	haystac
mod200e2repsp	178	35	107	71	0.6	39.9	haystac
mod200e3repgn	180	38	104	76	0.7	42.2	haystac
mod200e3repsp	178	35	107	71	0.6	39.9	haystac
mod200l1	176	31	103	73	0.6	41.5	haystac
anc200e1	176	204	104	72	3.7	40.9	kraken
anc200e2repgn	180	156	103	77	2.9	42.8	kraken
anc200e2repsp	178	198	106	72	3.6	40.4	kraken
anc200e3repgn	180	151	103	77	2.8	42.8	kraken
anc200e3repsp	178	201	106	72	3.7	40.4	kraken
anc200l1	176	114	102	74	2.1	42	kraken
mod200e1	176	256	104	72	4.7	40.9	kraken
mod200e2repgn	180	199	103	77	3.6	42.8	kraken
mod200e2repsp	178	262	106	72	4.8	40.4	kraken
mod200e3repgn	180	205	103	77	3.7	42.8	kraken
mod200e3repsp	178	262	106	72	4.8	40.4	kraken
mod200l1	176	161	103	73	2.9	41.5	kraken

anc200e1	176	154	104	72	2.8	40.9	malt
anc200e2repgn	180	129	102	78	2.4	43.3	malt
anc200e2repsp	178	152	105	73	2.8	41	malt
anc200e3repgn	180	130	102	78	2.4	43.3	malt
anc200e3repsp	178	154	105	73	2.8	41	malt
anc200l1	176	104	100	76	1.9	43.2	malt
mod200e1	176	172	104	72	3.1	40.9	malt
mod200e2repgn	180	154	102	78	2.8	43.3	malt
mod200e2repsp	178	173	105	73	3.2	41	malt
mod200e3repgn	180	153	102	78	2.8	43.3	malt
mod200e3repsp	178	170	105	73	3.1	41	malt
mod200l1	176	123	101	75	2.2	42.6	malt
anc200e1	176	44	103	73	0.8	41.5	sigma
anc200e2repgn	180	43	103	77	0.8	42.8	sigma
anc200e2repsp	178	43	106	72	0.8	40.4	sigma
anc200e3repgn	180	44	103	77	0.8	42.8	sigma
anc200e3repsp	178	43	106	72	0.8	40.4	sigma
anc200l1	176	36	102	74	0.7	42	sigma
mod200e1	176	31	103	73	0.6	41.5	sigma
mod200e2repgn	180	31	103	77	0.6	42.8	sigma
mod200e2repsp	178	28	106	72	0.5	40.4	sigma
mod200e3repgn	180	31	103	77	0.6	42.8	sigma
mod200e3repsp	178	27	106	72	0.5	40.4	sigma
mod200l1	176	29	102	74	0.5	42	sigma

Supplemental table S5. Mean species counts per genus per method for the genus database analysis.

Genus	Method	False Positive Count	False Negative Count	True Detected Species Count
Bacteroides	haystac	0	0	0.4
Bacteroides	kraken	23.6	0	0.4
Bacteroides	malt	22.7	0.1	0.3
Bacteroides	sigma	0	0	0.4

Burkholderia	haystac	0.5	0	0.2
Burkholderia	kraken	2.8	0	0.2
Burkholderia	malt	2.8	0	0.2
Burkholderia	sigma	0.8	0	0.2
Campylobacter	haystac	0	0.2	2.9
Campylobacter	kraken	19	0.1	3
Campylobacter	malt	19	0.1	3
Campylobacter	sigma	0	0.2	2.9
Clostridium	haystac	0.45	0	2.8
Clostridium	kraken	88.5	0.3	2.5
Clostridium	malt	61.8	0.6	2.2
Clostridium	sigma	0.85	0	2.8
Corynebacterium	haystac	0	0	3.3
Corynebacterium	kraken	53.05	0.1	3.2
Corynebacterium	malt	60.5	0.1	3.2
Corynebacterium	sigma	0	0.1	3.2
Desulfitobacterium	haystac	0	0	0.1
Desulfitobacterium	kraken	3.9	0	0.1
Desulfitobacterium	malt	3.9	0	0.1
Desulfitobacterium	sigma	0	0	0.1
Mycobacterium	haystac	0.5	0.5	1.1
Mycobacterium	kraken	34.15	0.35	1.25
Mycobacterium	malt	32.8	0.4	1.2
Mycobacterium	sigma	1.45	0.5	1.1
Solimonas	haystac	0	0	0.3
Solimonas	kraken	2.7	0	0.3
Solimonas	malt	2.7	0	0.3
Solimonas	sigma	0	0	0.3
Streptococcus	haystac	5.9	0	6.3
Streptococcus	kraken	70.45	0	6.3
Streptococcus	malt	55.7	0	6.3
Streptococcus	sigma	6.8	0	6.3

Supplemental table S6. Oral Microbiome dataset simulated species. A total of 196 species was simulated for the Oral Microbiome dataset. Only 115 species were included in the database, with only 4 of these species sharing a common accession.

Species	Simulated accessions	Database accessions
Abiotrophia defectiva	GCF_000160075.2	ACIN03
Actinomyces cardiffensis	GCF_000364865.1	AQHZ01
Actinomyces dentalis	NZ_AUBL00000000.1	AUBL01
Actinomyces massiliensis	GCF_000269805.1	AKIO01
Actinomyces oris	GCF_001553935.1	NZ_CP014232.1
Aggregatibacter actinomycetemcomitans	CP001733.2, NZ_ADOB00000000.2	NZ_CP012959.1
Alloprevotella tannerae	NZ_ACIJ00000000.2	ACIJ02
Anaeroglobus geminatus	GCF_000239275.1	AGCJ01
Atopobium parvulum	CP001721.1	NC_013203.1
Atopobium rimae	NZ_ACFE00000000.1	ACFE01
Bifidobacterium dentium	NZ_ABIX00000000.2	NZ_AP012326.1
Bordetella bronchiseptica	NC_002927.3	NZ_CP014013.1
Bordetella parapertussis	NC_018828.1	NC_018828.1
Bordetella pertussis	BX470248.1	NZ_CP017405.1
Campylobacter concisus	CP000792.2	NZ_CP012541.1
Campylobacter curvus	CP000767.2	NC_009715.2
Campylobacter gracilis	GCF_001190745.1	NZ_CP012196.1
Campylobacter showae	NZ_AMZQ00000000.1	AMZQ01
Capnocytophaga gingivalis	GCF_000174755.1	ACLQ01
Capnocytophaga granulosa	GCF_000411115.1	ADDA01
Capnocytophaga ochracea	GCF_000023285.1	NC_013162.1
Cardiobacterium valvarum	NZ_AGCM00000000.1	AGCM01
Catonella morbi	GCF_000160035.2	ACIL03
Clostridium botulinum	GCF_000020165.1	NC_010674.1
Clostridium tetani	GCF_000007625.1	NC_004557.1
Corynebacterium diphtheriae	BX248353.1	NZ_LN831026.1

Corynebacterium durum	GCF_000318135.1	AMEM01
Corynebacterium matruchotii	GCF_000175375.1	ACSH02
Corynebacterium urealyticum	AM942444.1	NC_010545.1
Dialister invisus	GCF_000160055.1	ACIM02
Eggerthella lenta	CP001726.1	NC_013204.1
Eikenella corrodens	GCF_000158615.1	ACEA01
Eubacterium brachy	GCF_000488855.1	AXUD01
Eubacterium saphenum	GCF_000161975.1	ACON01
Filifactor alocis	GCF_000163895.2	NC_016630.1
Finegoldia magna	GCF_000010185.1	NC_010376.1
Fusobacterium nucleatum	CP007062.1, AE009951.2	NZ_CP007062.1
Fusobacterium periodonticum	GCF_000158215.3	ACDC03
Gemella sanguinis	NZ_ACRY00000000.2	JNJO01
Granulicatella adiacens	GCF_000160675.1	ACKZ01
Granulicatella elegans	NZ_ACRF00000000.2	ACRF02
Haemophilus parainfluenzae	FQ312002.1	NZ_CP031477.1
Johnsonella ignava	GCF_000235445.1	ACZL01
Kingella denitrificans	NZ_AEWV00000000.1	AEWV01
Kingella oralis	NZ_ACJW00000000.2	ACJW02
Klebsiella pneumoniae	CP000964.1	NZ_WKJE00000000.1
Lachnoanaerobaculum saburreum	GCF_000185385.1	LSDA01
Lactobacillus brevis	CP000416.1	NC_008497.1
Leptotrichia buccalis	NC_013192.1, CP001685.1	NC_013192.1
Methanobrevibacter oralis	GCF_001639275.1	LWMU01
Mogibacterium timidum	GCF_000525775.1	JALU01
Mycobacterium avium	NC_008595.1, NC_021200.1	AGAQ01
Neisseria bacilliformis	GCF_000194925.1	AFAY01
Neisseria elongata	GCF_000818035.1	NZ_CP007726.1
Neisseria lactamica	GCF_000196295.1	NC_014752.1

Neisseria sicca	NZ_ACKO00000000.2	NZ_CP020452.2
Olsenella uli	GCF_000143845.1	NC_014363.1
Parvimonas micra	GCF_000800295.1	NZ_CP009761.1
Peptoniphilus lacrimalis	NZ_ADDO00000000.1	ARKX01
Peptostreptococcus anaerobius	NZ_ADJN00000000.1	ARMA01
Peptostreptococcus stomatis	GCF_000147675.1	ADGQ01
Porphyromonas asaccharolytica	NZ_AENO00000000.1	NC_015501.1
Porphyromonas endodontalis	GCF_000174815.1	ACNN01
Porphyromonas gingivalis	NC_010729.1, CP013131.1, CP012889.1, AP009380.1	NC_010729.1
Prevotella denticola	CP002589.1	NC_015311.1
Prevotella intermedia	NZ_JAEZ00000000.1, GCF_000261025.1	NZ_CP019301.1
Prevotella loescheii	GCF_000378085.1	ARJO01
Prevotella maculosa	NZ_ARNR00000000.1	ARNR01
Prevotella micans	NZ_BAKH00000000.1	BAKH01
Prevotella nigrescens	NZ_AFPX00000000.1	AFPX01
Prevotella oralis	NZ_AEPE00000000.2	AEPE02
Prevotella oris	NZ_BAJC00000000.1	BAJC01
Prevotella oulorum	NZ_ADGI00000000.1	BAJQ01
Prevotella saccharolytica	GCF_000614205.1	BAKN01
Prevotella salivae	AEQO01000001.1	AEQO01
Proteus mirabilis	NZ_ACLE00000000.1	NC_010554.1
Pseudomonas fluorescens	GCF_000237065.1	NC_016830.1
Pseudomonas putida	NC_015733.1	JBOP01
Pseudoramibacter alactolyticus	GCF_000185505.1	AEQN01
Pyramidobacter piscolens	GCF_000177335.1	ADFP01
Rothia dentocariosa	NC_014643.1, CP002280.1	NC_014643.1
Rothia mucilaginosa	NZ_ACVO00000000.1	NC_013715.1

Scardovia wiggsiae	NZ_AKCI00000000.1	AKCI01
Selenomonas sputigena	NZ_ACKP00000000.2	NC_015437.1
Shewanella denitrificans	GCF_000013765.1	NC_007954.1
Slackia exigua	GCF_000162875.1	ACUX02
Solobacterium moorei	NZ_AECQ00000000.1	AUKY01
Staphylococcus saprophyticus	NC_007350.1	JUTO01
Stomatobaculum longum	NZ_AGEL00000000.1	AGEL01
Streptococcus anginosus	NZ_NARD00000000.1	NC_022239.1
Streptococcus anginosus	NZ_NARD00000000.1	NZ_AP013072.1
Streptococcus gordonii	GCF_000017005.1	NC_009785.1
Streptococcus gordonii	GCF_000017005.1	NZ_CP012648.1
Streptococcus intermedius	AP010969.1	NC_022246.1
Streptococcus intermedius	AP010969.1	NZ_AP014880.1
Streptococcus mitis	FN568063.1	NZ_CP014326.1
Streptococcus oralis	GCF_000344275.1	NZ_CP023507.1
Streptococcus parasanguinis	CP002843.1	NC_015678.1
Streptococcus parasanguinis	CP002843.1	NC_017905.1
Streptococcus salivarius	FR873481.1	NZ_CP009913.1
Streptococcus salivarius	FR873481.1	NZ_CP020451.2
Streptococcus sanguinis	CP000387.1	NZ_APLR00000000.1
Streptococcus sobrinus	NZ_AGGP00000000.1	JMLC01
Streptococcus sobrinus	NZ_AGGP00000000.1	NZ_CP029490.1
Streptomyces scopuliridis	GCF_000718095.1	JOEI01
Streptomyces yeochonensis	GCF_000745345.1	JQNR01
Tannerella forsythia	GCF_000238215.1, NZ_MEHX00000000.1, NZ_FMML00000000.1, NZ_FMMM00000000.1, NZ_FMMN00000000.1	NC_016610.1
Treponema lecithinolyticum	NZ_AWVH00000000.1	AWVH01
Treponema maltophilum	GCF_000413055.1	ATFF01
Treponema socranskii	GCF_000413015.1	ATFD01

Treponema vincentii	NZ_ACYH00000000.1	ATFC01
Veillonella parvula	NZ_AEXI00000000.1, GCF_000024945.1, NZ_ADFU00000000.1, ASKE01000001.1	NC_013520.1
Vibrio vulnificus	GCF_000009745.1	NZ_CP014637.1
Yersinia pestis	NC_003143.1, CP000308.1	NZ_CP033699.1
Yersinia pseudotuberculosis	NC_010465.1	NZ_CP033713.1

Supplemental table S7. False positive species, whose RefSeq reference genomes contain human contamination.

False positive species	WGS accession	Accession containing human scaffolds
Paeniglutamicibacter antarcticus	CZJT01	yes
Alcanivorax hongdengensis	AMRJ01	yes
Sanguibacteroides justesenii	JPIU01	yes
Nocardia brevicatena	BAFU01	yes
Thioflexothrix psekupsii	MSLT01	yes
Gemmata obscuriglobus	ABGO01	yes

Supplemental table S8. Table of species simulated for the Oral Microbiome dataset. Samples containing an 'l' character in their names contain species in various abundances, whereas samples with an 'e' in their name contain species in constant abundances throughout. Genus negative control samples have the whole *Corynebacterium* genus removed, whereas species negative controls do not include *Corynebacterium diphtheriae*.

Species - Positive controls	l samples abundances	e samples abundances	Species - Negative controls genus	l_samples abundances	e_samples abundances	Species - Negative controls species	l samples abundances	e samples abundances
Abiotrophia defectiva	0.008	0.005	*Abiotrophia defectiva*	0.008	0.005	*Abiotrophia defectiva*	0.008	0.005
Actinobaculum sp oral taxon 183	0.006	0.005	*Actinobaculum sp oral taxon 183*	0.006	0.005	*Actinobaculum sp oral taxon 183*	0.006	0.005
Actinomyces cardiffensis	0.004	0.005	*Actinomyces cardiffensis*	0.004	0.005	*Actinomyces cardiffensis*	0.004	0.005
Actinomyces dentalis DSM 19115	0.03	0.005	*Actinomyces dentalis*	0.03	0.005	*Actinomyces dentalis*	0.03	0.005
Actinomyces massiliensis	0.003	0.005	*Actinomyces massiliensis*	0.003	0.005	*Actinomyces massiliensis*	0.003	0.005
Actinomyces oris	0.006	0.005	*Actinomyces oris*	0.006	0.005	*Actinomyces oris*	0.006	0.005
Actinomyces sp oral taxon 175	0.002	0.005	*Actinomyces sp oral taxon 170*	0.005	0.005	*Actinomyces sp oral taxon 175*	0.002	0.005
Actinomyces sp oral taxon 181	0.001	0.005	Actinomyces sp oral taxon 175	0.002	0.005	Actinomyces sp oral taxon 181	0.001	0.005
Actinomyces sp oral taxon 848	0.009	0.005	*Actinomyces sp oral taxon 178*	0.005	0.005	*Actinomyces sp oral taxon 848*	0.009	0.005
Aggregatibacter actinomycetemcomitans	0.007	0.01	*Actinomyces sp oral taxon 181*	0.001	0.005	*Aggregatibacter actinomycetemcomitans*	0.007	0.01

Aggregatibacter aphrophilus	0.009	0.005	*Actinomyces sp oral taxon 848*	0.009	0.005	*Aggregatibacter aphrophilus*	0.009	0.005
Aggregatibacter segnis	0.001	0.005	*Aggregatibacter actinomycetemcomitans*	0.007	0.01	*Aggregatibacter segnis*	0.001	0.005
Alloprevotella tannerae	0.007	0.005	*Aggregatibacter aphrophilus*	0.009	0.005	*Alloprevotella tannerae*	0.007	0.005
Anaeroglobus geminatus	0.004	0.005	*Aggregatibacter segnis*	0.001	0.005	*Anaeroglobus geminatus*	0.004	0.005
Atopobium parvulum	0.005	0.005	*Agrobacterium tumefaciens*	0.003	0.005	*Atopobium parvulum*	0.005	0.005
Atopobium rimae	0.003	0.005	*Alloprevotella tannerae*	0.007	0.005	*Atopobium rimae*	0.003	0.005
Atopobium sp oral taxon 810	0.002	0.005	*Anaeroglobus geminatus*	0.004	0.005	*Atopobium sp oral taxon 810*	0.002	0.005
Bacillus subtilis	0.001	0.005	*Atopobium parvulum*	0.005	0.005	*Bacillus subtilis*	0.001	0.005
Bacteroidetes bacterium oral taxon 272	0.005	0.005	Atopobium rimae	0.003	0.005	*Bacteroidetes bacterium oral taxon 272*	0.005	0.005
Bacteroidetes oral taxon 274	0.002	0.005	*Atopobium sp oral taxon 810*	0.002	0.005	*Bacteroidetes oral taxon 274*	0.002	0.005
Bifidobacterium dentium	0.006	0.005	*Bacillus subtilis*	0.001	0.005	*Bifidobacterium dentium*	0.006	0.005
Bifidobacterium longum	0.001	0.005	*Bacteroidetes bacterium oral taxon 272*	0.005	0.005	Bifidobacterium longum	0.001	0.005
Bordetella bronchiseptica	0.004	0.005	*Bacteroidetes oral taxon 274*	0.002	0.005	*Bordetella bronchiseptica*	0.004	0.005
Bordetella parapertussis	0.001	0.005	*Bifidobacterium dentium*	0.006	0.005	*Bordetella parapertussis*	0.001	0.005
Bordetella pertussis	0.001	0.005	*Bifidobacterium longum*	0.001	0.005	*Bordetella pertussis*	0.001	0.005
Burkholderia mallei	0.001	0.005	*Bordetella bronchiseptica*	0.004	0.005	*Burkholderia mallei*	0.001	0.005

Burkholderia pseudomallei	0.001	0.005	*Bordetella parapertussis*	0.001	0.005	*Burkholderia pseudomallei*	0.001	0.005
Campylobacter concisus	0.005	0.005	*Bordetella pertussis*	0.001	0.005	*Campylobacter concisus*	0.005	0.005
Campylobacter curvus	0.002	0.005	*Burkholderia mallei*	0.001	0.005	*Campylobacter curvus*	0.002	0.005
Campylobacter gracilis	0.002	0.005	Burkholderia pseudomallei	0.001	0.005	*Campylobacter gracilis*	0.002	0.005
Campylobacter rectus	0.004	0.005	*Campylobacter concisus*	0.005	0.005	*Campylobacter rectus*	0.004	0.005
Campylobacter showae	0.005	0.005	*Campylobacter curvus*	0.002	0.005	*Campylobacter showae*	0.005	0.005
Candidate division SR1 bacterium MGEHA 2517589202 0	0.008	0.005	*Campylobacter gracilis*	0.002	0.005	*Candidate division SR1 bacterium MGEHA 2517589202 0*	0.008	0.005
Candidate division TM7 single cell isolate TM7a	0.002	0.005	*Campylobacter rectus*	0.004	0.005	*Candidate division TM7 single cell isolate TM7a*	0.002	0.005
candidate division TM7 single cell isolate TM7b	0.006	0.005	*Campylobacter showae*	0.005	0.005	*candidate division TM7 single cell isolate TM7b*	0.006	0.005
candidate division TM7 single cell isolate TM7c	0.003	0.005	*Candidate division SR1 bacterium MGEHA 2517589202 0*	0.008	0.005	candidate division TM7 single cell isolate TM7c	0.003	0.005
Capnocytophaga gingivalis	0.01	0.005	*Candidate division TM7 single cell isolate TM7a*	0.002	0.005	*Capnocytophaga gingivalis*	0.01	0.005
Capnocytophaga granulosa	0.002	0.005	*candidate division TM7 single cell isolate TM7b*	0.006	0.005	*Capnocytophaga granulosa*	0.002	0.005
Capnocytophaga ochracea	0.002	0.005	*candidate division TM7 single cell isolate TM7c*	0.003	0.005	*Capnocytophaga ochracea*	0.002	0.005

Capnocytophaga sp oral taxon 329	0.005	0.005	*Capnocytophaga gingivalis*	0.01	0.005	*Capnocytophaga sp oral taxon 329*	0.005	0.005
Capnocytophaga sp oral taxon 332	0.004	0.005	Capnocytophaga granulosa	0.002	0.005	*Capnocytophaga sp oral taxon 332*	0.004	0.005
Capnocytophaga sputigena	0.004	0.005	*Capnocytophaga ochracea*	0.002	0.005	*Capnocytophaga sputigena*	0.004	0.005
Cardiobacterium hominis	0.003	0.005	*Capnocytophaga sp oral taxon 329*	0.005	0.005	*Cardiobacterium hominis*	0.003	0.005
Cardiobacterium valvarum	0.009	0.005	*Capnocytophaga sp oral taxon 332*	0.004	0.005	*Cardiobacterium valvarum*	0.009	0.005
Catonella morbi	0.01	0.005	*Capnocytophaga sputigena*	0.004	0.005	*Catonella morbi*	0.01	0.005
Clostridium botulinum	0.001	0.005	*Cardiobacterium hominis*	0.003	0.005	*Clostridium botulinum*	0.001	0.005
Clostridium tetani	0.005	0.005	*Cardiobacterium valvarum*	0.009	0.005	*Clostridium tetani*	0.005	0.005
Corynebacterium diphtheriae	0.001	0.005	*Catonella morbi*	0.01	0.005	*Klebsiella pneumoniae*	0.001	0.005
Corynebacterium durum	0.01	0.005	*Clostridium botulinum*	0.001	0.005	*Corynebacterium durum*	0.01	0.005
Corynebacterium matruchotii	0.01	0.005	*Clostridium tetani*	0.005	0.005	Corynebacterium matruchotii	0.01	0.005
Corynebacterium urealyticum	0.005	0.005	*Desulfobulbus sp oral taxon 041*	0.01	0.005	*Corynebacterium urealyticum*	0.005	0.005
Desulfobulbus sp oral taxon 041	0.01	0.005	Dialister invisus	0.009	0.005	*Desulfobulbus sp oral taxon 041*	0.01	0.005
Dialister invisus	0.009	0.005	*Eggerthella lenta*	0.002	0.005	*Dialister invisus*	0.009	0.005

Eggerthella lenta	0.002	0.005	*Eikenella corrodens*	0.01	0.005	*Eggerthella lenta*	0.002	0.005
Eikenella corrodens	0.01	0.005	*Eubacterium brachy*	0.002	0.005	*Eikenella corrodens*	0.01	0.005
Eubacterium brachy	0.002	0.005	*Eubacterium saphenum*	0.001	0.005	*Eubacterium brachy*	0.002	0.005
Eubacterium saphenum	0.001	0.005	*Eubacterium yurii*	0.005	0.005	*Eubacterium saphenum*	0.001	0.005
Eubacterium yurii	0.005	0.005	*Filifactor alocis*	0.01	0.005	*Eubacterium yurii*	0.005	0.005
Filifactor alocis	0.01	0.005	*Finegoldia magna*	0.007	0.005	*Filifactor alocis*	0.01	0.005
Finegoldia magna	0.007	0.005	*Fretibacterium fastidiosum*	0.007	0.005	*Finegoldia magna*	0.007	0.005
Fretibacterium fastidiosum	0.007	0.005	*Fusobacterium nucleatum*	0.035	0.02	*Fretibacterium fastidiosum*	0.007	0.005
Fusobacterium nucleatum	0.035	0.02	*Fusobacterium periodonticum*	0.005	0.005	*Fusobacterium nucleatum*	0.035	0.02
Fusobacterium periodonticum	0.005	0.005	Gemella morbillorum	0.002	0.005	*Fusobacterium periodonticum*	0.005	0.005
Gemella morbillorum	0.002	0.005	*Gemella sanguinis*	0.009	0.005	Gemella morbillorum	0.002	0.005
Gemella sanguinis	0.009	0.005	*Granulicatella adiacens*	0.009	0.005	*Gemella sanguinis*	0.009	0.005
Granulicatella adiacens	0.009	0.005	*Granulicatella elegans*	0.002	0.005	*Granulicatella adiacens*	0.009	0.005
Granulicatella elegans	0.002	0.005	*Haemophilus ducreyi*	0.001	0.005	*Granulicatella elegans*	0.002	0.005
Haemophilus parainfluenzae	0.006	0.005	*Haemophilus parainfluenzae*	0.006	0.005	*Haemophilus parainfluenzae*	0.006	0.005
Johnsonella ignava	0.001	0.005	*Johnsonella ignava*	0.001	0.005	*Johnsonella ignava*	0.001	0.005
Kingella denitrificans	0.004	0.005	*Kingella denitrificans*	0.004	0.005	*Kingella denitrificans*	0.004	0.005
Kingella oralis	0.007	0.005	*Kingella kingeae*	0.005	0.005	*Kingella oralis*	0.007	0.005
Klebsiella pneumoniae	0.003	0.005	*Kingella oralis*	0.007	0.005	*Klebsiella pneumoniae*	0.003	0.005

Lachnoanaerobaculum saburreum	0.01	0.005	*Klebsiella pneumoniae*	0.004	0.01	*Lachnoanaerobaculum saburreum*	0.01	0.005
Lachnospiraceae oral taxon 107	0.005	0.005	Lachnoanaerobaculum saburreum	0.01	0.005	*Lachnospiraceae oral taxon 107*	0.005	0.005
Lactobacillus brevis	0.002	0.005	*Lachnospiraceae oral taxon 107*	0.005	0.005	*Lactobacillus brevis*	0.002	0.005
Lautropia mirabilis	0.01	0.005	*Lactobacillus acidophilus*	0.001	0.005	*Lautropia mirabilis*	0.01	0.005
Leptotrichia buccalis	0.006	0.005	*Lactobacillus brevis*	0.002	0.005	*Leptotrichia buccalis*	0.006	0.005
Leptotrichia sp oral taxon 215	0.002	0.005	*Lactobacillus salivarius*	0.01	0.005	Leptotrichia sp oral taxon 215	0.002	0.005
Methanobrevibacter unclassified	0.03	0.005	*Lautropia mirabilis*	0.01	0.005	*Methanobrevibacter unclassified*	0.03	0.005
Mogibacterium sp CM50	0.01	0.005	*Leptotrichia buccalis*	0.006	0.005	*Mogibacterium sp CM50*	0.01	0.005
Mycobacterium avium	0.009	0.01	*Leptotrichia shahii*	0.004	0.005	*Mycobacterium avium*	0.009	0.01
Mycobacterium bovis	0.005	0.005	*Leptotrichia sp oral taxon 215*	0.002	0.005	*Mycobacterium bovis*	0.005	0.005
Mycobacterium smegmatis	0.003	0.005	*Methanobrevibacter unclassified*	0.03	0.005	*Mycobacterium smegmatis*	0.003	0.005
Mycobacterium tuberculosis	0.001	0.005	*Microbacterium sp oral taxon 186*	0.001	0.005	*Haemophilus ducreyi*	0.001	0.005
Neisseria bacilliformis	0.001	0.005	Mogibacterium sp CM50	0.01	0.005	*Neisseria bacilliformis*	0.001	0.005
Neisseria elongata	0.007	0.005	*Neisseria bacilliformis*	0.001	0.005	*Neisseria elongata*	0.007	0.005
Neisseria lactamica	0.02	0.005	*Neisseria elongata*	0.007	0.005	*Neisseria lactamica*	0.02	0.005
Neisseria sicca	0.009	0.005	*Neisseria gonorrhoeae*	0.001	0.005	*Neisseria sicca*	0.009	0.005

Olsenella uli	0.007	0.005	*Neisseria lactamica*	0.02	0.005	*Olsenella uli*	0.007	0.005
Parvimonas micra	0.008	0.005	*Neisseria meningitidis*	0.01	0.005	*Parvimonas micra*	0.008	0.005
Parvimonas sp oral taxon 110	0.001	0.005	*Neisseria sicca*	0.009	0.005	*Parvimonas sp oral taxon 110*	0.001	0.005
Parvimonas sp oral taxon 393	0.004	0.005	*Olsenella uli*	0.007	0.005	Parvimonas sp oral taxon 393	0.004	0.005
Peptoniphilus lacrimalis	0.003	0.005	*Parvimonas micra*	0.008	0.005	*Peptoniphilus lacrimalis*	0.003	0.005
Peptoniphilus sp oral taxon 386	0.002	0.005	*Parvimonas sp oral taxon 110*	0.001	0.005	*Peptoniphilus sp oral taxon 386*	0.002	0.005
Peptostreptococcus anaerobius	0.005	0.005	*Parvimonas sp oral taxon 393*	0.004	0.005	*Peptostreptococcus anaerobius*	0.005	0.005
Peptostreptococcus stomatis	0.002	0.005	Peptoniphilus lacrimalis 315 B	0.003	0.005	*Peptostreptococcus stomatis*	0.002	0.005
Porphyromonas asaccharolytica	0.006	0.005	*Peptoniphilus sp oral taxon 386*	0.002	0.005	*Porphyromonas asaccharolytica*	0.006	0.005
Porphyromonas endodontalis	0.008	0.005	*Peptostreptococcus anaerobius*	0.005	0.005	*Porphyromonas endodontalis*	0.008	0.005
Porphyromonas gingivalis	0.016	0.02	*Peptostreptococcus stomatis*	0.002	0.005	*Porphyromonas gingivalis*	0.016	0.02
Porphyromonas sp oral taxon 278	0.004	0.005	*Porphyromonas asaccharolytica*	0.006	0.005	*Porphyromonas sp oral taxon 278*	0.004	0.005
Prevotella denticola	0.004	0.005	*Porphyromonas endodontalis*	0.008	0.005	*Prevotella denticola*	0.004	0.005
Prevotella intermedia	0.008	0.01	*Porphyromonas gingivalis*	0.016	0.02	*Prevotella intermedia*	0.008	0.01

Prevotella loescheii	0.002	0.005	*Porphyromonas sp oral taxon 278*	0.004	0.005	*Prevotella loescheii*	0.002	0.005
Prevotella maculosa	0.001	0.005	*Prevotella denticola*	0.004	0.005	*Prevotella maculosa*	0.001	0.005
Prevotella melaninogenica	0.004	0.005	*Prevotella intermedia*	0.008	0.01	*Prevotella melaninogenica*	0.004	0.005
Prevotella micans	0.003	0.005	*Prevotella loescheii*	0.002	0.005	Prevotella micans	0.003	0.005
Prevotella nigrescens	0.007	0.005	Prevotella maculosa	0.001	0.005	*Prevotella nigrescens*	0.007	0.005
Prevotella oralis	0.02	0.005	*Prevotella marshii*	0.001	0.005	*Prevotella oralis*	0.02	0.005
Prevotella oris	0.002	0.005	*Prevotella melaninogenica*	0.004	0.005	*Prevotella oris*	0.002	0.005
Prevotella oulorum	0.002	0.005	*Prevotella micans*	0.003	0.005	*Prevotella oulorum*	0.002	0.005
Prevotella saccharolytica	0.001	0.005	*Prevotella nigrescens*	0.007	0.005	*Prevotella saccharolytica*	0.001	0.005
Prevotella salivae	0.001	0.005	*Prevotella oralis*	0.02	0.005	*Prevotella salivae*	0.001	0.005
Prevotella sp oral taxon 299	0.004	0.005	*Prevotella oris*	0.002	0.005	*Prevotella sp oral taxon 299*	0.004	0.005
Prevotella sp oral taxon 317	0.004	0.005	*Prevotella oulorum*	0.002	0.005	*Prevotella sp oral taxon 317*	0.004	0.005
Prevotella sp oral taxon 473	0.003	0.005	*Prevotella saccharolytica*	0.001	0.005	*Prevotella sp oral taxon 473*	0.003	0.005
Propionibacterium propionicum	0.005	0.005	*Prevotella salivae*	0.001	0.005	*Propionibacterium propionicum*	0.005	0.005
Proteus mirabilis	0.002	0.005	*Prevotella sp oral taxon 299*	0.004	0.005	*Proteus mirabilis*	0.002	0.005
Pseudomonas aeruginosa	0.001	0.005	Prevotella sp oral taxon 317	0.004	0.005	*Pseudomonas aeruginosa*	0.001	0.005

Pseudomonas fluorescens	0.001	0.005	*Prevotella sp oral taxon 473*	0.003	0.005	*Pseudomonas fluorescens*	0.001	0.005
Pseudomonas putida	0.001	0.005	*Prevotella veroralis*	0.001	0.005	Pseudomonas putida	0.001	0.005
Pseudomonas syringae	0.004	0.005	*Propionibacterium avidum*	0.001	0.005	*Pseudomonas syringae*	0.004	0.005
Pseudoramibacter alactolyticus	0.003	0.005	*Propionibacterium propionicum*	0.005	0.005	*Pseudoramibacter alactolyticus*	0.003	0.005
Pyramidobacter piscolens	0.007	0.005	*Proteus mirabilis*	0.002	0.005	*Pyramidobacter piscolens*	0.007	0.005
Rothia aeria	0.005	0.005	*Pseudomonas aeruginosa*	0.001	0.005	*Rothia aeria*	0.005	0.005
Rothia dentocariosa	0.011	0.01	*Pseudomonas fluorescens*	0.001	0.005	*Rothia dentocariosa*	0.011	0.01
Rothia mucilaginosa	0.002	0.005	*Pseudomonas putida*	0.001	0.005	*Rothia mucilaginosa*	0.002	0.005
Salmonella enterica	0.004	0.02	*Pseudomonas syringae*	0.004	0.005	*Lactobacillus acidophilus*	0.001	0.005
Scardovia wiggsiae	0.002	0.005	*Pseudoramibacter alactolyticus*	0.003	0.005	*Salmonella enterica*	0.003	0.015
Selenomonas flueggei	0.004	0.005	Pyramidobacter piscolens	0.007	0.005	*Scardovia wiggsiae*	0.002	0.005
Selenomonas noxia	0.007	0.005	*Rothia aeria*	0.005	0.005	*Selenomonas flueggei*	0.004	0.005
Selenomonas sp oral taxon 138	0.003	0.005	*Rothia dentocariosa*	0.011	0.01	*Selenomonas noxia*	0.007	0.005
Selenomonas sp oral taxon 149	0.002	0.005	*Rothia mucilaginosa*	0.002	0.005	*Selenomonas sp oral taxon 138*	0.003	0.005
Selenomonas sputigena	0.009	0.005	*Scardovia wiggsiae*	0.002	0.005	*Selenomonas sp oral taxon 149*	0.002	0.005
Serratia odorifera	0.001	0.005	*Selenomonas artemidis*	0.004	0.005	Selenomonas sputigena	0.009	0.005

Shewanella denitrificans	0.001	0.005	*Selenomonas flueggei*	0.004	0.005	*Serratia odorifera*	0.001	0.005
Slackia exigua	0.004	0.005	*Selenomonas noxia*	0.007	0.005	*Shewanella denitrificans*	0.001	0.005
Solobacterium moorei	0.01	0.005	*Selenomonas sp oral taxon 138*	0.003	0.005	*Slackia exigua*	0.004	0.005
Staphylococcus aureus	0.003	0.005	*Selenomonas sp oral taxon 149*	0.002	0.005	*Solobacterium moorei*	0.01	0.005
Staphylococcus epidermidis	0.002	0.005	*Selenomonas sputigena*	0.009	0.005	*Staphylococcus aureus*	0.003	0.005
Staphylococcus saprophyticus	0.001	0.005	Serratia odorifera	0.001	0.005	*Staphylococcus epidermidis*	0.002	0.005
Stomatobaculum longum	0.009	0.005	*Shewanella denitrificans*	0.001	0.005	*Staphylococcus saprophyticus*	0.001	0.005
Streptococcus anginosus	0.004	0.005	*Slackia exigua*	0.004	0.005	*Stomatobaculum longum*	0.009	0.005
Streptococcus constellatus	0.001	0.005	*Solobacterium moorei*	0.01	0.005	*Streptococcus anginosus*	0.004	0.005
Streptococcus gordonii	0.008	0.005	*Staphylococcus aureus*	0.003	0.005	*Streptococcus constellatus*	0.001	0.005
Streptococcus intermedius	0.002	0.005	*Staphylococcus epidermidis*	0.002	0.005	*Streptococcus gordonii*	0.008	0.005
Streptococcus mitis	0.006	0.005	*Staphylococcus saprophyticus*	0.001	0.005	*Streptococcus intermedius*	0.002	0.005
Streptococcus oligofermentans	0.002	0.005	*Stomatobaculum longum*	0.009	0.005	*Streptococcus mitis*	0.006	0.005
Streptococcus oralis	0.03	0.005	*Streptococcus anginosus*	0.004	0.005	Streptococcus oligofermentans	0.002	0.005

Streptococcus parasanguinis	0.001	0.005	*Streptococcus constellatus*	0.001	0.005	*Streptococcus oralis*	0.03	0.005
Streptococcus salivarius	0.004	0.005	*Streptococcus gordonii*	0.008	0.005	*Streptococcus parasanguinis*	0.001	0.005
Streptococcus sanguinis	0.005	0.005	Streptococcus intermedius	0.002	0.005	*Streptococcus salivarius*	0.004	0.005
Streptococcus sobrinus	0.002	0.005	*Streptococcus mitis*	0.006	0.005	*Streptococcus sanguinis*	0.005	0.005
Streptococcus sp oral taxon 056	0.006	0.005	*Streptococcus oligofermentans*	0.002	0.005	*Streptococcus sobrinus*	0.002	0.005
Streptococcus sp oral taxon 058	0.002	0.005	*Streptococcus oralis*	0.03	0.005	*Streptococcus sp oral taxon 056*	0.006	0.005
Streptomyces kanamyceticus	0.001	0.005	*Streptococcus parasanguinis*	0.001	0.005	*Streptococcus sp oral taxon 058*	0.002	0.005
Streptomyces scopuliridis	0.004	0.005	*Streptococcus salivarius*	0.004	0.005	*Streptomyces kanamyceticus*	0.001	0.005
Streptomyces yeochonensis	0.002	0.005	*Streptococcus sanguinis*	0.005	0.005	*Streptomyces scopuliridis*	0.004	0.005
Tannerella forsythia	0.062	0.025	*Streptococcus sobrinus*	0.002	0.005	*Streptomyces yeochonensis*	0.002	0.005
Tissierellia bacterium	0.015	0.01	*Streptococcus sp oral taxon 056*	0.006	0.005	*Tannerella forsythia*	0.062	0.025
Treponema denticola	0.018	0.015	*Streptococcus sp oral taxon 058*	0.002	0.005	*Tissierellia bacterium*	0.015	0.01
Treponema lecithinolyticum	0.003	0.005	*Streptomyces kanamyceticus*	0.001	0.005	*Treponema denticola*	0.018	0.015

Treponema maltophilum	0.004	0.005	Streptomyces scopuliridis	0.004	0.005	Treponema lecithinolyticum	0.003	0.005
Treponema socranskii	0.008	0.005	*Streptomyces yeochonensis*	0.002	0.005	*Treponema maltophilum*	0.004	0.005
Treponema vincentii	0.002	0.005	*Tannerella forsythia*	0.062	0.025	*Treponema socranskii*	0.008	0.005
Veillonella atypica	0.006	0.005	*Tissierellia bacterium*	0.015	0.01	*Treponema vincentii*	0.002	0.005
Veillonella denticariosi	0.002	0.005	*Treponema denticola*	0.018	0.015	*Veillonella atypica*	0.006	0.005
Veillonella dispar	0.009	0.005	*Treponema lecithinolyticum*	0.003	0.005	*Veillonella denticariosi*	0.002	0.005
Veillonella oral taxon 780	0.001	0.005	*Treponema maltophilum*	0.004	0.005	*Veillonella dispar*	0.009	0.005
Veillonella parvula	0.015	0.02	*Treponema pallidum*	0.001	0.005	*Veillonella oral taxon 780*	0.001	0.005
Veillonella sp oral taxon 158	0.003	0.005	*Treponema socranskii*	0.008	0.005	*Veillonella parvula*	0.015	0.02
Vibrio cholerae	0.001	0.005	*Treponema vincentii*	0.002	0.005	*Veillonella sp oral taxon 158*	0.003	0.005
Vibrio vulnificus	0.002	0.005	*Veillonella atypica*	0.006	0.005	*Vibrio cholerae*	0.001	0.005
Xanthomonas axonopodis	0.002	0.005	Veillonella denticariosi	0.002	0.005	*Vibrio vulnificus*	0.002	0.005
Yersinia enterocolitica	0.001	0.005	*Veillonella dispar*	0.009	0.005	*Xanthomonas axonopodis*	0.002	0.005
Yersinia pestis	0.002	0.01	*Veillonella oral taxon 780*	0.001	0.005	*Yersinia enterocolitica*	0.001	0.005
Yersinia pseudotuberculosis YPIII	0.004	0.005	*Veillonella parvula*	0.015	0.02	Neisseria gonorrhoeae	0.001	0.005
			Veillonella sp oral taxon 158	0.003	0.005	*Microbacterium sp oral taxon 186*	0.001	0.005

			Vibrio cholerae	0.001	0.005	*Yersinia pseudotuberculosis*	0.004	0.005
			Vibrio vulnificus	0.002	0.005			
			Xanthomonas axonopodis	0.002	0.005			

Supplemental table S9. Memory and time usage data for database construction across HAYSTAC, Kraken2/Bracken, MALT and Sigma.

Database (species)	Method	Max RSS (KB)	Runtime	Runtime_s
100	HAYSTAC	4456836	03:24.5	204.52
500	HAYSTAC	16459260	15:34.4	934.42
10	HAYSTAC	399736	00:19.2	19.21
100	HAYSTAC	4459024	03:24.0	204.04
500	HAYSTAC	16459184	15:33.1	933.09
10	HAYSTAC	399508	00:16.4	16.41
500	HAYSTAC	16459144	15:41.6	941.58
100	HAYSTAC	4460828	03:26.1	206.14
10	HAYSTAC	402396	00:16.0	15.96
100	HAYSTAC	4448636	03:24.6	204.56
500	HAYSTAC	16459200	15:44.1	944.1
10	HAYSTAC	396636	00:16.7	16.71
100	HAYSTAC	4435036	03:34.0	214.01
500	HAYSTAC	16457152	16:17.1	977.06
10	HAYSTAC	401016	00:16.6	16.61
100	Kraken2/Bracken	18533948	08:27.6	507.56
10	Kraken2/Bracken	17322584	06:18.5	378.52
500	Kraken2/Bracken	23033944	14:29.0	869.03
100	Kraken2/Bracken	18533744	08:06.3	486.33
10	Kraken2/Bracken	17322652	06:35.5	395.45
500	Kraken2/Bracken	23033904	15:04.8	904.76
100	Kraken2/Bracken	18533996	08:36.7	516.69
10	Kraken2/Bracken	17322584	06:39.0	399.05
500	Kraken2/Bracken	23033896	14:50.7	890.67
100	Kraken2/Bracken	18534088	08:19.6	499.59
10	Kraken2/Bracken	17322420	06:30.8	390.75
500	Kraken2/Bracken	23033832	15:04.1	904.11
100	Kraken2/Bracken	18533924	08:31.0	510.95
10	Kraken2/Bracken	17322432	06:29.8	389.84
500	Kraken2/Bracken	23033780	14:36.2	876.22

100	MALT	8882660	03:36.3	216.27
10	MALT	4311824	00:36.6	36.59
500	MALT	36382652	13:31.6	811.61
100	MALT	8840420	03:21.6	201.64
10	MALT	4315812	00:36.0	36.04
500	MALT	31800464	14:10.2	850.23
100	MALT	8862576	03:18.2	198.16
10	MALT	4310920	00:34.9	34.88
500	MALT	31840612	13:32.6	812.56
100	MALT	8864660	03:35.8	215.81
10	MALT	4265280	00:35.0	34.96
500	MALT	36309684	13:44.0	824
100	MALT	8816548	03:40.1	220.11
10	MALT	4314312	00:35.0	34.96
500	MALT	36358480	13:15.3	795.33
500	SIGMA	120528	08:23.7	503.67
100	SIGMA	118232	01:42.4	102.38
10	SIGMA	106356	00:11.7	11.75
500	SIGMA	126924	08:25.9	505.92
100	SIGMA	120388	01:42.3	102.28
10	SIGMA	106264	00:11.7	11.67
500	SIGMA	119896	08:24.5	504.47
100	SIGMA	119284	01:42.0	102
10	SIGMA	106304	00:11.7	11.65
500	SIGMA	120732	08:23.7	503.75
100	SIGMA	118856	01:42.1	102.1
10	SIGMA	106468	00:11.6	11.63
500	SIGMA	119320	08:24.2	504.15
100	SIGMA	126936	01:42.4	102.36
10	SIGMA	106288	00:11.6	11.64

Supplemental table S10. Memory and time usage data for sample analysis across HAYSTAC, Kraken2/Bracken, MALT and Sigma.

Number of Reads	Database (species)	Method	Max RSS (KB)	Runtime	Runtime_s
10000	500	HAYSTAC	2753616	17:15.5	1035.46
100000	500	HAYSTAC	2876520	12:36.2	756.15
1000000	100	HAYSTAC	868356	03:43.8	223.83
1000000	500	HAYSTAC	2979588	18:17.6	1097.6
1000000	10	HAYSTAC	761948	01:00.9	60.87
10000	500	HAYSTAC	2754828	13:12.2	792.17
100000	500	HAYSTAC	2888300	13:46.1	826.07
1000000	100	HAYSTAC	868168	03:48.5	228.53
1000000	500	HAYSTAC	2975288	18:28.6	1108.57
1000000	10	HAYSTAC	762368	01:12.8	72.83
10000	500	HAYSTAC	2752936	13:18.5	798.51
100000	500	HAYSTAC	2883752	13:29.4	809.4
1000000	100	HAYSTAC	867848	03:46.4	226.38
1000000	500	HAYSTAC	2977404	18:20.7	1100.68
1000000	10	HAYSTAC	759968	01:05.9	65.86
10000	500	HAYSTAC	2754788	13:18.9	798.88
100000	500	HAYSTAC	2880068	12:30.0	750
1000000	100	HAYSTAC	914076	03:39.6	219.56
1000000	500	HAYSTAC	2978772	17:36.5	1056.46
1000000	10	HAYSTAC	765604	01:02.2	62.18
10000	500	HAYSTAC	2759116	13:25.5	805.5
100000	500	HAYSTAC	2880148	14:20.2	860.24
1000000	100	HAYSTAC	887688	03:47.6	227.59
1000000	500	HAYSTAC	2981104	20:57.2	1257.21
1000000	10	HAYSTAC	755680	01:03.6	63.56
1000000	500	Kraken2/Bracken	4231408	00:02.9	2.87
1000000	500	Kraken2/Bracken	4302984	00:02.9	2.9
1000000	10	Kraken2/Bracken	1067884	00:02.0	1.98
10000	500	Kraken2/Bracken	3340872	00:01.3	1.32
100000	500	Kraken2/Bracken	3430460	00:01.4	1.41

1000000	100	Kraken2/Bracken	1669348	00:02.3	2.32
1000000	500	Kraken2/Bracken	4260680	00:02.9	2.85
1000000	500	Kraken2/Bracken	4357048	00:03.0	2.96
1000000	10	Kraken2/Bracken	1076696	00:02.1	2.06
10000	500	Kraken2/Bracken	3342856	00:01.3	1.29
100000	500	Kraken2/Bracken	3421400	00:01.4	1.43
1000000	100	Kraken2/Bracken	1815116	00:02.8	2.79
1000000	500	Kraken2/Bracken	4347080	00:03.0	3
1000000	500	Kraken2/Bracken	4284224	00:03.0	2.96
1000000	10	Kraken2/Bracken	1080904	00:02.0	1.99
10000	500	Kraken2/Bracken	3342208	00:01.3	1.26
100000	500	Kraken2/Bracken	3447968	00:01.4	1.44
1000000	100	Kraken2/Bracken	1937628	00:02.8	2.8
1000000	500	Kraken2/Bracken	4293160	00:03.0	2.97
1000000	500	Kraken2/Bracken	4439392	00:02.9	2.93
1000000	10	Kraken2/Bracken	1081460	00:02.0	2
10000	500	Kraken2/Bracken	3341240	00:01.3	1.25
100000	500	Kraken2/Bracken	3446604	00:01.4	1.44
1000000	100	Kraken2/Bracken	1748036	00:02.6	2.6
1000000	500	Kraken2/Bracken	4203620	00:02.9	2.88
1000000	500	Kraken2/Bracken	4341496	00:02.9	2.93
1000000	10	Kraken2/Bracken	1037572	00:02.0	1.97
10000	500	Kraken2/Bracken	3341208	00:01.3	1.27
100000	500	Kraken2/Bracken	3433264	00:01.4	1.41
1000000	100	Kraken2/Bracken	1814736	00:02.6	2.64
1000000	10	MALT	5677696	00:37.2	37.2
1000000	500	MALT	50907728	06:12.6	372.62
100000	500	MALT	39809708	02:49.9	169.95
1000000	100	MALT	19279776	02:28.7	148.74
10000	500	MALT	39089116	02:28.1	148.11
1000000	10	MALT	5374428	00:36.8	36.85
1000000	500	MALT	49155612	06:17.0	377.04
100000	500	MALT	39562592	02:51.6	171.63
1000000	100	MALT	19214652	02:26.8	146.81
10000	500	MALT	38408528	02:27.5	147.49

1000000	10	MALT	5412664	00:36.8	36.77
1000000	500	MALT	50151000	06:14.5	374.54
100000	500	MALT	40261828	02:49.3	169.26
1000000	100	MALT	19466508	02:26.9	146.93
10000	500	MALT	39117120	02:34.6	154.6
1000000	10	MALT	5380460	00:36.5	36.5
1000000	500	MALT	49048656	06:14.2	374.21
100000	500	MALT	39608712	02:49.8	169.77
1000000	100	MALT	19748944	02:30.0	149.96
10000	500	MALT	38399292	02:29.2	149.17
1000000	10	MALT	5530436	01:10.1	70.15
1000000	500	MALT	49308400	06:15.2	375.19
100000	500	MALT	40141984	02:50.4	170.39
1000000	100	MALT	18872220	02:27.8	147.81
10000	500	MALT	38390228	02:28.1	148.1
1000000	10	SIGMA	22280	00:22.4	22.42
1000000	500	SIGMA	31360	09:03.9	543.94
10000	500	SIGMA	31192	08:30.8	510.79
100000	500	SIGMA	30916	08:36.9	516.85
1000000	100	SIGMA	29760	02:01.5	121.47
1000000	10	SIGMA	21196	00:22.6	22.63
1000000	500	SIGMA	29652	09:10.3	550.33
10000	500	SIGMA	31080	08:35.0	515.03
100000	500	SIGMA	30980	08:41.2	521.16
1000000	100	SIGMA	29940	02:02.8	122.78
1000000	10	SIGMA	21972	00:22.5	22.48
1000000	500	SIGMA	31400	09:06.1	546.08
10000	500	SIGMA	29548	08:33.9	513.88
100000	500	SIGMA	31516	08:38.9	518.93
1000000	100	SIGMA	29892	02:02.4	122.41
1000000	10	SIGMA	21252	00:22.5	22.47
1000000	500	SIGMA	30996	09:07.9	547.87
10000	500	SIGMA	29396	08:33.4	513.35
100000	500	SIGMA	31760	08:37.7	517.69
1000000	100	SIGMA	31660	02:01.8	121.8

1000000	10	SIGMA	21992	00:22.4	22.39
1000000	500	SIGMA	29608	09:07.9	547.92
10000	500	SIGMA	29156	08:34.5	514.51
100000	500	SIGMA	31192	08:38.9	518.86
1000000	100	SIGMA	29996	02:03.2	123.2

Supplemental table S11. Pathogenic and non-pathogenic species used for the pathogen detection analysis.

Genus	Non-pathogenic	Pathogenic
Clostridium	*Clostridium aceticum*	*Clostridium tetani*
Prevotella	*Prevotella bivia*	*Prevotella intermedia*
Bacteroides	*Bacteroides coprosuis*	*Bacteroides pyogenes*
Corynebacterium	*Corynebacterium durum*	*Corynebacterium diphtheriae*
Streptococcus	*Streptococcus cristatus*	*Streptococcus pneumoniae*
Lactobacillus	*Lactobacillus equi*	*Lactobacillus hominis*
Ruminococcus	*Ruminococcus faecis*	*Ruminococcus bromii*
Eubacterium	*Eubacterium limosum*	*Eubacterium ventriosum*
Mycobacterium	*Mycobacterium conspicuum*	*Mycobacterium haemophilum*
Actinomyces	*Actinomyces meyeri*	*Actinomyces dentalis*
Mycoplasma	*Mycoplasma salivarium*	*Mycoplasma suis*
Acinetobacter	*Acinetobacter apis*	*Acinetobacter baumannii*
Staphylococcus	*Staphylococcus simulans*	*Staphylococcus haemolyticus*
Helicobacter	*Helicobacter hepaticus*	*Helicobacter heilmannii*
Leptospira	*Leptospira broomii*	*Leptospira kirschneri*
Porphyromonas	*Porphyromonas asaccharolytica*	*Porphyromonas gingivalis*
Bifidobacterium	*Bifidobacterium breve*	*Bifidobacterium dentium*
Campylobacter	*Campylobacter fetus*	*Campylobacter jejuni*
Alistipes	*Alistipes inops*	*Alistipes obesi*
Neisseria	*Neisseria canis*	*Neisseria mucosa*
Roseburia	*Roseburia hominis*	*Roseburia faecis*
Nocardia	*Nocardia brasiliensis*	*Nocardia asteroides*
Brachyspira	*Brachyspira alvinipulli*	*Brachyspira hyodysenteriae*
Peptoniphilus	*Peptoniphilus asaccharolyticus*	*Peptoniphilus urinimassiliensis*

Anaerococcus	*Anaerococcus lactolyticus*	*Anaerococcus vaginalis*
Blautia	*Blautia hydrogenotrophica*	*Blautia obeum*
Capnocytophaga	*Capnocytophaga canis*	*Capnocytophaga canimorsus*
Fusobacterium	*Fusobacterium ulcerans*	*Fusobacterium nucleatum*
Dialister	*Dialister invisus*	*Dialister pneumosintes*
Parabacteroides	*Parabacteroides chartae*	*Parabacteroides distasonis*
Bartonella	*Bartonella henselae*	*Bartonella bovis*
Coprococcus	*Coprococcus comes*	*Coprococcus eutactus*
Dorea	*Dorea longicatena*	*Dorea formicigenerans*
Leptotrichia	*Leptotrichia goodfellowii*	*Leptotrichia buccalis*
Paenibacillus	*Paenibacillus beijingensis*	*Paenibacillus polymyxa*
Selenomonas	*Selenomonas ruminantium*	*Selenomonas sputigena*
Atopobium	*Atopobium parvulum*	*Atopobium vaginae*
Haemophilus	*Haemophilus parahaemolyticus*	*Haemophilus influenzae*
Treponema	*Treponema putidum*	*Treponema pallidum*
Yersinia	*Yersinia pseudotuberculosis*	*Yersinia pestis*
Enterococcus	*Enterococcus faecalis*	*Enterococcus casseliflavus*
Moraxella	*Moraxella ovis*	*Moraxella catarrhalis*
Desulfovibrio	*Desulfovibrio piger*	*Desulfovibrio desulfuricans*
Listeria	*Listeria booriae*	*Listeria rocourtiae*
Pseudomonas	*Streptomyces cinnamoneus*	*Streptomyces scabiei*
Bordetella	*Bordetella bronchiseptica*	*Bordetella pertussis*
Brevibacterium	*Brevibacterium linens*	*Brevibacterium casei*
Ehrlichia	*Ehrlichia ruminantium*	*Ehrlichia canis*
Klebsiella	*Klebsiella aerogenes*	*Klebsiella oxytoca*
Legionella	*Legionella adelaidensis*	*Legionella clemsonensis*
Vibrio	*Vibrio alginolyticus*	*Vibrio vulnificus*
Acetobacter	*Acetobacter aceti*	*Acetobacter cibinongensis*
Chlamydia	*Chlamydia felis*	*Chlamydia suis*
Pseudomonas	*Pseudomonas putida*	*Pseudomonas fluorescens*
Rickettsia	*Rickettsia felis*	*Rickettsia typhi*
Deinococcus	*Deinococcus puniceus*	*Deinococcus proteolyticus*
Shewanella	*Shewanella baltica*	*Shewanella algae*
Nocardiopsis	*Nocardiopsis flavescens*	*Nocardiopsis dassonvillei*
Halomonas	*Halomonas huangheensis*	*Halomonas elongata*

Bacillus	*Bacillus simplex*	*Bacillus licheniformis*
Mycolicibacterium	*Mycolicibacterium goodii*	*Mycolicibacterium aurum*
Chryseobacterium	*Chryseobacterium scophthalmum*	*Chryseobacterium indologenes*
Weissella	*Weissella viridescens*	*Weissella cibaria*
Acholeplasma	*Acholeplasma laidlawii*	*Acholeplasma brassicae*
Arcobacter	*Arcobacter butzleri*	*Arcobacter cryaerophilus*
Comamonas	*Comamonas kerstersii*	*Comamonas testosteroni*
Cellulomonas	*Cellulomonas fimi*	*Cellulomonas massiliensis*
Pediococcus	*Pediococcus pentosaceus*	*Pediococcus acidilactici*
Providencia	*Providencia sneebia*	*Providencia stuartii*
Kingella	*Kingella denitrificans*	*Kingella kingae*
Gemella	*Gemella asaccharolytica*	*Gemella haemolysans*
Cutibacterium	*Cutibacterium avidum*	*Cutibacterium acnes*
Devosia	*Devosia psychrophila*	*Devosia chinhatensis*
Tenacibaculum	*Tenacibaculum dicentrarchi*	*Tenacibaculum maritimum*
Borreliella	*Borreliella bissettii*	*Borreliella valaisiana*
Gordonia	*Gordonia polyisoprenivorans*	*Gordonia bronchialis*
Pedobacter	*Pedobacter steynii*	*Pedobacter heparinus*
Sphingobium	*Sphingobium baderi*	*Sphingobium yanoikuyae*
Arthrobacter	*Arthrobacter nitrophenolicus*	*Arthrobacter crystallopoietes*
Oceanobacillus	*Oceanobacillus iheyensis*	*Oceanobacillus picturae*
Stenotrophomonas	*Stenotrophomonas acidaminiphila*	*Stenotrophomonas maltophilia*
Pasteurella	*Pasteurella skyensis*	*Pasteurella multocida*
Spiroplasma	*Spiroplasma mirum*	*Spiroplasma turonicum*
Veillonella	*Veillonella magna*	*Veillonella parvula*
Aerococcus	*Aerococcus christensenii*	*Aerococcus urinae*
Actinobaculum	*Actinobaculum massiliense*	*Actinobaculum suis*
Sutterella	*Sutterella parvirubra*	*Sutterella wadsworthensis*
Mobiluncus	*Mobiluncus curtisii*	*Mobiluncus mulieris*
Myroides	*Myroides profundi*	*Myroides injenensis*
Oribacterium	*Oribacterium sinus*	*Oribacterium parvum*
Sphingobacterium	*Sphingobacterium deserti*	*Sphingobacterium spiritivorum*
Odoribacter	*Odoribacter laneus*	*Odoribacter splanchnicus*
Scardovia	*Scardovia inopinata*	*Scardovia wiggsiae*

Trueperella	*Trueperella bernardiae*	*Trueperella pyogenes*
Rothia	*Rothia dentocariosa*	*Rothia mucilaginosa*
Massilia	*Massilia putida*	*Massilia timonae*
Kluyvera	*Kluyvera cryocrescens*	*Kluyvera ascorbata*
Pantoea	*Pantoea alhagi*	*Pantoea agglomerans*
Cetobacterium	*Cetobacterium ceti*	*Cetobacterium somerae*
Xanthomonas	*Xanthomonas citri*	*Xanthomonas massiliensis*