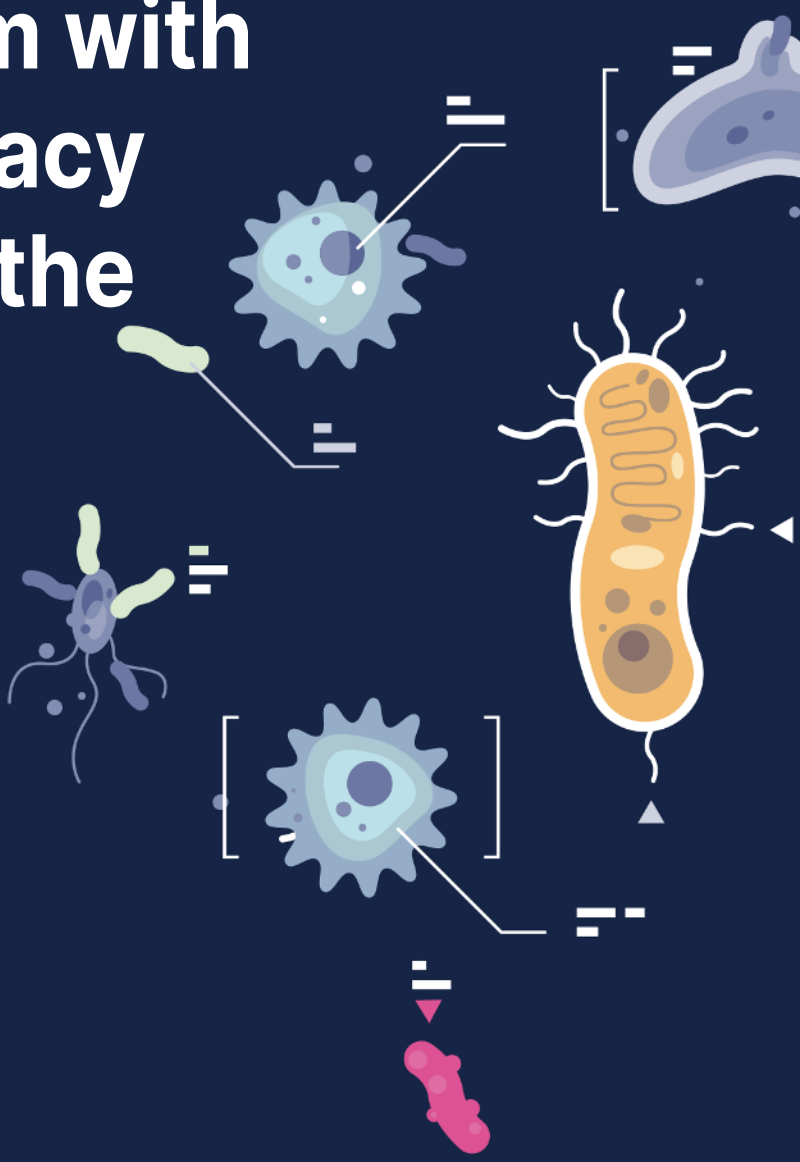
COSMOSID®
Unlocking The Microbiome

An Interactive Metagenomics Analysis Platform with Increased Accuracy and Precision at the Strain-level



CosmosID-HUB: An Interactive Metagenomics Analysis Platform with Increased Accuracy and Precision at the Strain-level

Introduction:

The advent of metagenomic sequencing has led to significant advancements in our understanding of the microbiome in a wide variety of contexts, from the human body[1] to farm animals[2,3], soil, and marine environments[4]. Each unique environment provides its own set of challenges in accurate microbial identification, including low microbial biomass in ocean samples, PCR inhibitors in soil samples, and high host DNA in certain human and animal samples. Sequencing of the 16S rRNA gene has been a popular and low-cost way to identify microbes in these samples. However, this method is limited by PCR primer and amplification bias along with unreliable identification below the genus level[5]. 16S rRNA gene sequencing studies that use primer sets targeting different variable regions cannot be compared directly as different regions selectively detect different bacterial taxa[6]. Further, relative abundance measures are inaccurate due to variation in the number of 16S rRNA gene operons present within differing bacterial species[7].

Whole genome shotgun sequencing (WGS) addresses the amplification, primer bias, and relative quantification issues by avoiding amplification altogether and sequencing the entire bacterial genome. Here, unique, single-copy marker gene sequences or reference genome sequences can be used to identify and quantify bacteria present within a sample more accurately. Because WGS relies on marker genes or alignment to a reference, it is possible to accurately identify microorganisms at the species or even strain taxonomic levels. However, there is wide variation in the accuracy of computational tools developed to perform these microbial identifications. Challenges faced in WGS methods include the requirement to account for variations in genetic diversity within species (i.e. some species are very diverse, whereas others are genetically uniform), mobile elements that are shared among species, the quality of reference genomes used, and the divergence of strains found in nature from the reference genomes that are used for identification. Here, we perform a benchmarking study to evaluate the performance of CosmosID-HUB to five other publicly available taxonomic classification algorithms Centrifuge[11], Metaphlan3[12], Kraken2_Bracken[13], mOTUs2[29] and Metalign[30]. These publicly available taxonomic classification algorithms are known for its high accuracy and precision when compared to other publicly available methods based on previous benchmarking evaluations[8-10].An ideal metagenomics classifier will properly identify a large number of microorganisms while displaying a small number of false positives at all taxonomic levels. For this study, we used publicly available benchmarking datasets from CAMI2(Mouse Gut Dataset)[27] and *McIntyre et al* 2017 benchmarking paper[28], which consisted of mock communities of known compositions, to perform these comparisons.

Importance of Strain Level Resolution

As metagenomics is increasingly becoming a method of choice across multi-disciplinary applications, the importance of sub-species and strain level variation is becoming ever more apparent.[14-23] For example, specific strains of *Streptococcus mutans* produce hemorrhagic damage in the murine brain and other tissues[18], whereas other strains are risk factors for

ulcerative colitis.[17] Likewise, different strains of the protozoan parasite *Toxoplasma gondii* manifest diverse pathologies and elicit altered host responses[19]. Particular variants of *Staphylococcus epidermidis*[15] and *Staphylococcus aur*eus[16] affect virulence and biofilm formation. Certain strains of *Bifidobacterium longum*, but not others, protect against pathogens like *Escherichia coli*, and still others elicit differential immunomodulatory properties.[14] Similarly, strain-specific immunomodulatory effects are seen for *Propionibacterium freudenreichii*[21] and for another probiotic agent, *Lactobacillus casei*, variants derived from different ecological niches vary in their ability to bind foodborne carcinogens.[22] The importance of strain resolution is much more apparent when assigning attribution, as exemplified in outbreaks of nosocomial infections such as *Legionella pneumophila*[23-24] and *Klebsiella pneumoniae*.[25] These examples serve to underscore why sub-species and strain level identification is so crucial to our understanding of microbial symbiosis and dysbiosis, and thus demonstrate the power of CosmosID-HUB metagenomics in defining the microbiome composition at a finer taxonomic resolution – critical information needed in microbiome research, epidemiological studies, microbial forensics, and outbreak investigations.

Evaluation of the CosmosID-HUB Taxonomic Profiler:

In this study, we compared the performance of the CosmosID-HUB taxonomic profiling algorithm (CosmosID-HUB), to that of Centrifuge[11], Metaphlan3[12], Kraken2_Bracken[13], mOTUs2[29] and Metalign[30]

To evaluate the performance of these tools, we used datasets of known microbial composition from CAMI2 and *McIntyre et al* 2017 benchmarking papers respectively to determine true positives, false positives, and false negatives from each of the pipelines. While tools can produce either very aggressive or highly conservative predictions of community composition, to be reliably used in multi-disciplinary microbiome applications it is critical that overall classification accuracy and detection resolution of a tool maintain low rates of false positives and false negatives. Therefore, we evaluated the recall or sensitivity of the results (fraction of species actually present in the metagenomes that are correctly detected) and the precision (fraction of species identified that were actually included in the mock community). Since there is often a trade-off between precision and sensitivity, we also calculated the F1 score, which is the harmonic mean of sensitivity and precision which helps to evaluate both metrics in one score. In figure 1, the performance of the evaluated tools is compared at different taxonomic levels. CosmosID-HUB had the highest F1 score, outperforming the other tools at both species and strain levels. On recall at species level (Figure 2), Kraken2_Bracken and Centrifuge outperformed CosmosID-HUB for the *McIntyre et al* 2017 benchmarking dataset, but at the cost of a very high number of false positives (Figure 5). This is reflected in the precision metric (Figure 3), where both Kraken2_Bracken and Centrifuge performed poorly. Similarly, Metaphlan3 performed better on precision but at the cost of recall. Since CosmosID-HUB's performance across precision and recall was similar, the F1 score clearly reflects CosmosID-HUB's superior performance in correctly identifying the right taxa in the dataset while keeping the false positives low. Lastly, at

strain level resolution, CosmosID-HUB was clearly superior and outperformed Kraken2_Bracken across F1 score, precision, and recall. Centrifuge, Metaphlan3 Metalign and mOTUs2 were unable to make strain-level calls.

The public tools that are used in this evaluation fall into two different categories; 1) DNA to DNA based methods (Kraken2_Bracken, Centrifuge, and Metalign) that compares sequencing reads with an exhaustive collection of whole prokaryotic genomes. 2) DNA to marker gene based methods (Metaphlan3 and mOTUs2) that query only specific gene families or clade specific biomarkers in their reference databases to the sequencing reads. Being able to compare the sequencing reads to the entire genome allows a unique advantage of discriminating among different strains within a certain species. However, it is extremely challenging to carry out strain level analysis primarily because of short reads mapping to multiple genomes due to either local or global homology between different species and within species as well. That's one of the primary reasons why only kraken2_Bracken was able to go down to strain level whereas the rest of DNA to DNA methods (Centrifuge and Metalign) were unable to. For DNA to marker gene based methods, they are unable to go down to strain level resolution since their database is structured in a manner that consists of specific marker genes per clade. Furthermore, in recent years, the authors of Metaphlan3 have developed a companion tool called StrainPhlAn[12] which utilizes the marker gene sequences in Metaphlan3 database and analyzes the variants within these markers to genotype the strains of each species. However StrainPhlAn is limited to genotyping only the most abundant species and the identification of only the most abundant strain per species in a community, since detecting multiple strains per species requires much greater depth (well above 10X). CosmosID-HUB's unique approach to taxonomic profiling allows it to take full advantage of the entire genome of prokaryotes as well as focus on unique signatures/biomarkers per strain to accurately and precisely discriminate between different strains even at lower coverage than 10X.

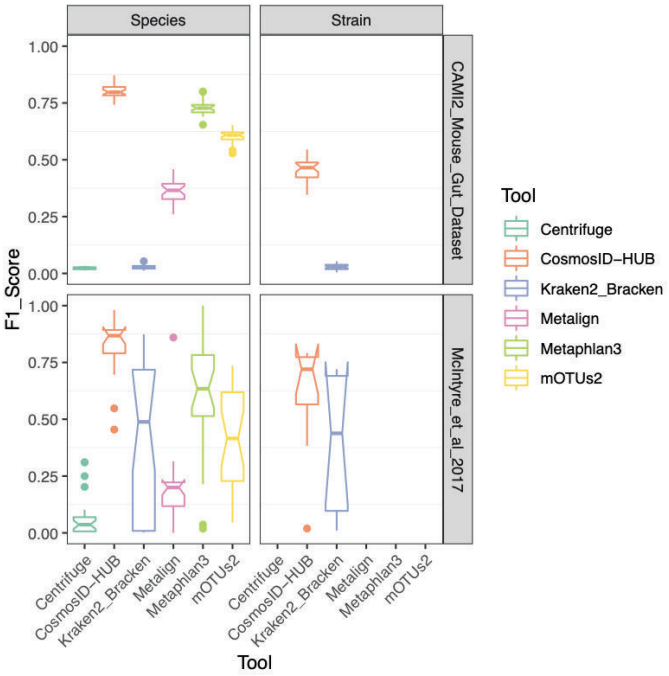


Figure 1: F1 Scores at the Species and Strain Levels. F1 scores were calculated for data analyzed using Centrifuge, CosmosID-HUB, Kraken2_Bracken, Metalign, Metaphlan3 and mOTUs2 at the (A) species, and (B) strain levels. CosmosID-HUB outperforms all the other pipelines at all taxonomic levels.

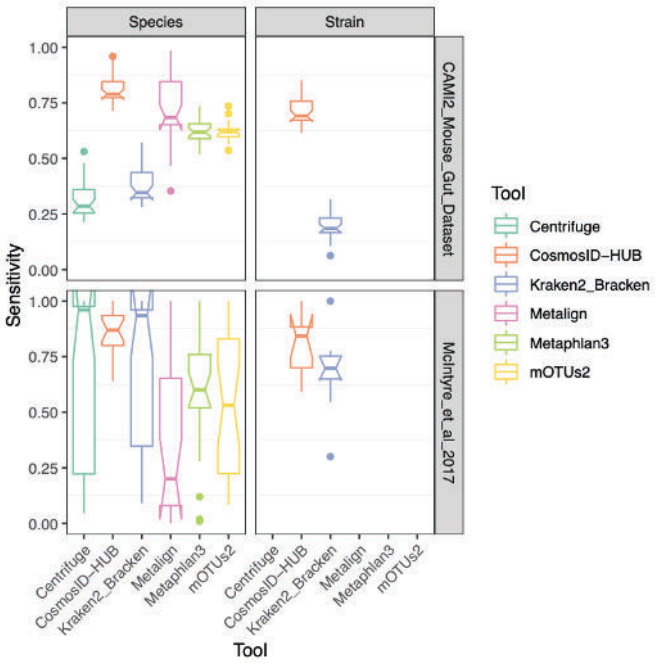


Figure 2: Sensitivity Scores at the Species, and Strain Levels. Sensitivity was calculated at the (A) species, and (B) strain levels. CosmosID-HUB has the highest sensitivity and second highest sensitivity at species level for CAMI2 Mouse Gut and McIntyre et al 2017 datasets respectively. At strain level, CosmosID-HUB has higher sensitivity compared to Kraken2_Bracken

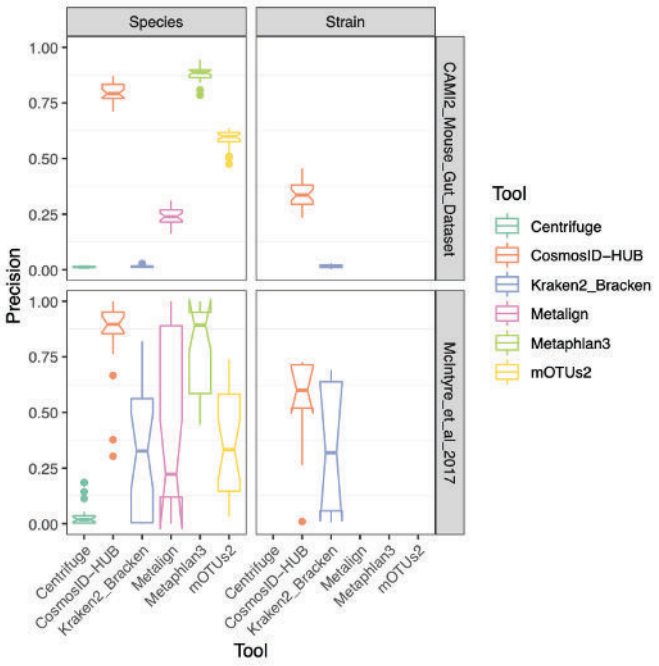


Figure 3: Precision Scores at the Species, and Strain Levels. Precision was calculated for all four pipelines at the (A) species, and (B) strain levels. Only Metaphlan3 barely outperforms CosmosID-HUB here.

Conclusion and Future Direction:

Overall, at species and strain levels, CosmosID-HUB performs better than Centrifuge, Kraken2_Bracken, Metaphlan3, Metalign and mOTUs2 across all evaluation metrics and particularly on the combined F1 score (the harmonic mean of sensitivity and precision). It's important to note here that, except Kraken2_Bracken, all the other remaining tools are unable to identify taxa to the strain level. The primary reason why most of the taxonomic profilers are unable to go down to strain level is because of short reads mapping to multiple genomes due to either local or global homology within the same species and different species as well. CosmosID-HUB's unique ability to differentiate between core and shared biomarkers among different prokaryotic genomes allows it to discriminate among strains of the same species accurately and precisely.

As the field of microbial genomics is advancing rapidly, we are actively working on R&D projects and will have some exciting new updates coming to CosmosID-HUB in the coming months. As sequencing is getting cheaper, we are getting exposed to a huge volume of sequencing data from different specimen types which gives us a great opportunity to understand the complexities and nuances with each communities/ecological niches individually. Using the huge collection of data available in the public domain for different specimen types, our R&D team is working on creating an advanced specimen type specific filters to further increase our accuracy and precision across the entire spectrum of microbiome niches starting from high complexity stool and soil specimens to low complexity vaginal and clinical specimens. Furthermore, with the rapid advancements in Long-Read sequencing

technology both in terms of cost and error rate, we are also actively working on a dedicated pipeline to support both long reads classification and MAGs/Genome Reconstruction which will allow us to unravel the microbial complexity with the highest resolution and precision ever! Lastly, in this age of modern high throughput “omics'' technologies ranging from metagenomics, metatranscriptomics to metaproteomics, metabolomics, CosmosID-HUB is dedicated to develop a unifying and comprehensive analysis platform where you can combine and aggregate all these data types with its associated clinical and experimental metadata and turn them into actionable insights for your relevant translational and basic science research. Few highlights of this application will involve slicing and dicing the Omics data with Multi-Omics approach using artificial intelligence, machine learning, advanced statistical models, and co-occurrence networks etcetera."

How to Cite Us and Algorithm Appendix:
Please refer to our Literature web page (https://www.cosmosid.com/literature/) to cite the previous peer-reviewed publications that have used the CosmosID-HUB Taxonomic Profiling algorithm for their microbiome related research.

The CosmosID-HUB taxonomic profiling algorithm has two separable comparators: the first consists of a pre-computation phase for the reference database and a per-sample computation. The input to the pre-computation phase is a comprehensive curated collection of reference microbial genomes and its output is a phylogeny tree, together with sets of variable length k-mer fingerprints (biomarkers) that are uniquely identified with distinct nodes, branches and leaves of the tree.

The second per-sample, computational phase searches the hundreds of millions of short sequence reads or contigs from draft assembly against the k-mer fingerprint sets. The resulting statistics are analyzed to give fine-grain composition and relative abundance estimates. The second comparator uses edit distance-scoring techniques to compare a target genome with a reference set. Overall classification precision is maintained through aggregation statistics. Enhanced detection specificity is achieved by running the comparators in sequence.

The first comparator finds reads in which there is an exact match with a k-mer uniquely identified with a reference genome; the second comparator then statistically scores the entire read against the reference to verify that the read is indeed uniquely identified with that reference. High-performance bioinformatics enables CosmosID to deliver speed and accuracy in microbial identification. The omprehensive curation collection of reference microbial genomes structured in a unique tree-like database structure provide extremely fine resolution in identification, discrimination of pathogens from ‘near-neighbours’, and accurate measurement of relative abundance. The user-friendly and secure cloud interface offers simplicity in operation, ease of use (without requiring bioinformatics skills), built-in multivariate comparative analysis and advanced statistical analysis modules to allow users to gain informative insights from their metagenomics datasets.

Evaluation Methods:

Dataset Sources

Publicly available metagenomics benchmarking datasets from CAMI2[27] (https://repository.publisso.de/resource/frl:6421672) and *McIntyre et al* 2017 paper28 which consisted of mock communities of known compositions were used for this study

Sample Processing Through Each Pipeline

The dataset has been ran through Centrifuge[11], Metaphlan3[12], Metalign[30], mOTUs2[29], Kraken2_Bracken[13] and ComosID_HUB Microbiome (https://app.cosmosid.com) with default databases and parameters.

Result files were automatically grouped by connecting the ground truth files to the corresponding output files from Centrifuge, Metaphlan3, Metalign, mOTUs2, Kraken2_Bracken and CosmosID-HUB Microbiome. The ground truth files in tsv format provided the NCBI Taxonomy IDs for species, and strain level per call. The result files were then parsed into python dictionaries and sets accordingly for downstream use.

Downloading NCBI Taxonomy Browser Database

NCBI Taxonomy Browser Database files were downloaded using wget through NCBI's File Transfer Protocol (FTP) site. Nodes.dmp was parsed into a python dictionary to allow quick identification of Taxonomy Rank given an NCBI Taxonomy ID.

Defining and Calculating Base Statistics

Python 2.7 set operations are noncommutative and were leveraged in the calculation of base statistics. Our base statistics are True Positives, False Positives, True Negatives, False Negatives are defined as follows:

True Positive (TP) - For a given tool, a call is a true positive if a corresponding entry is in the ground truth. True positives were calculated by taking the size of a resultant set. The resultant set was calculated through the intersection of a truth and result set of NCBI Taxonomy IDs.

False Positive (FP) - For a given tool, a call is a false positive if a corresponding entry is not in the ground truth. False positives were calculated by taking the size of a resultant set. The false positives were found by calculating the set difference of the result set and truth set.

False Negative (FN) - For a given tool, a false negative exists if no call is made for a corresponding entry in the ground truth. False negatives were calculated by taking the size of a resultant set. The false negatives were found by calculating the set difference of the truth set and result set.

True Negative (TN) - True Negatives were calculated by subtracting the calls made (TP + FP) from the total number of possible calls for a tool and taxonomy rank being evaluated. The total number of possible calls was calculated by parsing the DB files for a given tool and use of the Taxonomy browser dictionary where appropriate.

Supplementary Statistics

The calculation of our supplementary statistics were dependent on base statistic values.
Our supplementary statistics were F1, Sensitivity, and Precision were calculated with the following formulas:

Sensitivity: TPR=TP/(TP+FN)
Precision: PPV=TP/(TP+FP)
F1Score: F1=2TP/(2TP+FP+FN)

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Supplementary Section:

The remaining 3 figures and its associated text will be part of the supplementary section after References.

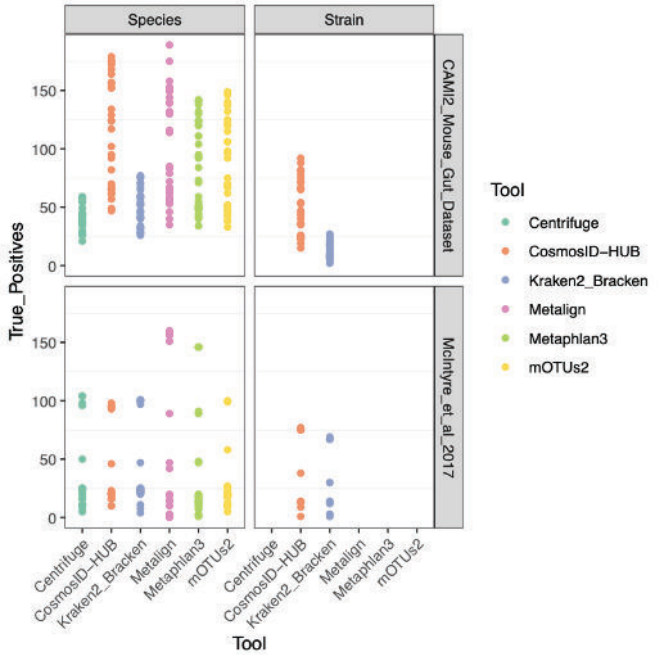

Figure 4: True Positive Counts for all Pipelines at the Species and Strain Level. True positive (TP) counts were calculated at the (A) species, and (B) strain level. Though not always the highest, the TP counts for the CosmosID-HUB are consistently high.

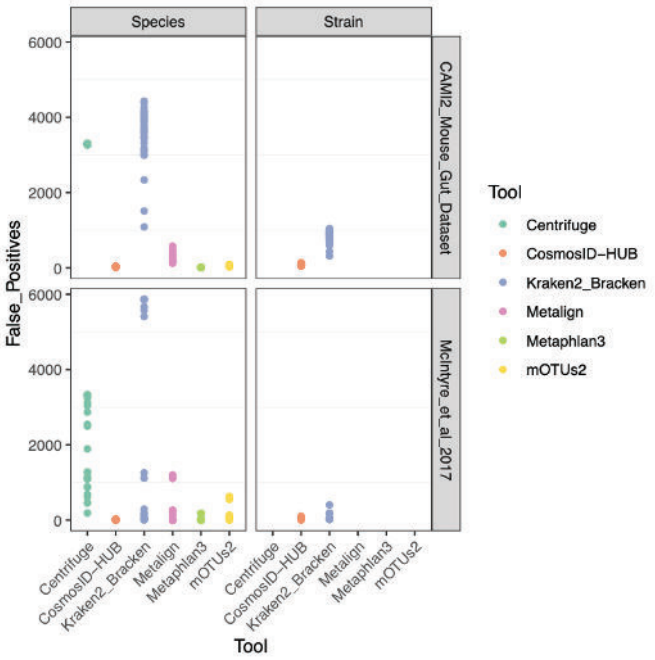

Figure 5: False Positive Counts for all Pipelines at the Species and Strain Levels. False positive (FP) counts were calculated at the (A) species, and (B) strain levels. CosmosID-HUB has consistently very low rates of FPs at all taxonomic levels.

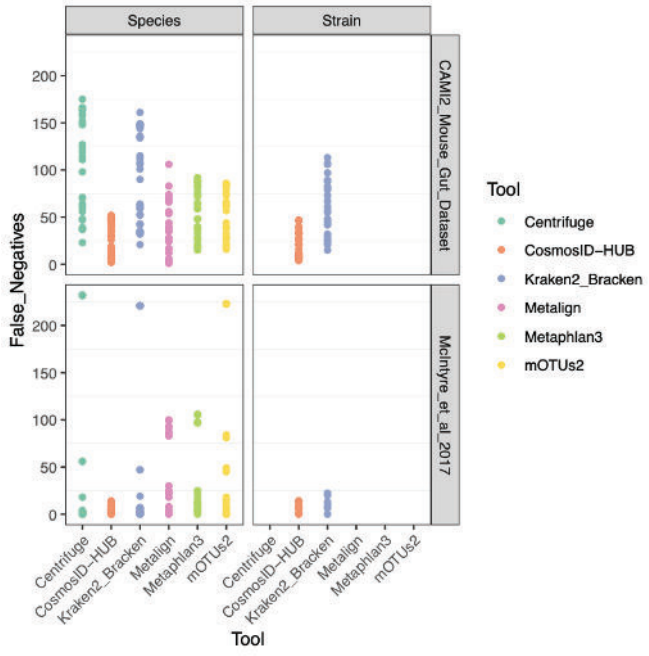

Figure 6: False Negative Counts for all Pipelines at Species, and Strain Levels. False negative (FN) counts were calculated at the (A) species, and (B) strain levels. CosmosID-HUB maintains low FN counts across all taxonomic levels.

At the species level, all four pipelines have similar true positive (TP) counts (Figure 4) at each taxonomic level with Metalign having the highest TPs. At the strain level, CosmosID-HUB has the highest TPs, highlighting it's superiority in making strain level classifications. In terms of false positive (FP) counts, Kraken2_Bracken has the highest false positive (FP) counts (Figure 5) at both species and strain level. Furthermore, CosmosID-HUB, as well as Metaphlan3, have close to zero FP counts at all taxonomic levels. Lastly, for false negatives counts (FN), CosmosID-HUB had the lowest number of FNs (Figure 6) at all taxonomic levels across both benchmarking datasets.