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Selection of software and database for metagenomics sequence analysis impacts the outcome of microbial profiling and pathogen detection

**Abbreviated running headline**

Impact of software selection on metagenomics

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**Abstract**

**Aim**

Shotgun metagenomic sequencing and analysis are widely used for microbial profiling of biological specimens and pathogen detection. However, very little is known about the technical biases caused by the choice of analysis software and databases. In this study, we evaluated popular shotgun metagenomics taxonomical profiling software to characterize the microbial compositions of biological samples collected from wild rodents.

**Method and Results**

Using nine of the most widely used metagenomics software and four different databases, we analyzed shotgun metagenomic sequence data from three sets of wild rodent tissue samples. We demonstrated the discrepancies in results when different databases and software were used, which cause significant variation in microbial communintiy characterizations. Our analysis also showed that these software differed in their ability to detect the presence of *Leptospira,* a major zoonotic pathogen of one health importance.

**Conclusions**

Significant differences in compositional profiles for the same dataset while using different databases and software combinations can result in confounding biological conclusions in microbial profiling.

**Significance and Impact of Study**

This study cautions that the selection of software and databases to analyze metagenomics data may influence the outcome and biological interpretation.

**Keywords**

shotgun metagenomic sequencing, pathogen detection, *Leptospira*, next-generation sequencing, software, databases, taxonomical profiles

**Introduction**

Studies analyzing the composition of microbial communities are frequently utilized in diverse study fields, such as ecology (Galbraith *et al.*, 2018; Grossart *et al.*, 2020), agriculture (Mashiane *et al.*, 2017; Granjou and Phillips, 2019), human and animal health (Chen *et al.*, 2019; Tun *et al.*, 2012; Zhong *et al.*, 2019), and pharmacology (Chavira *et al.*, 2019; Wang *et al.*, 2019). Traditional methods used to identify the microbial agents within a biological specimen include methods such as culture (Handelsman, 2004), antigen detection (Desmonts and Remington, 1980; Lequin, 2005), and nucleic acid detection (Yang and Rothman, 2004; Driscoll, 2009) protocols. However, these laboratory methods are limited to studying a single pathogen of interest and lack the ability to scrutinize the community of microorganisms potentially present in a sample. Next-Generation Sequencing (NGS) technologies have provided researchers with a set of culture-independent tools that identify pathogens directly from DNA sequences (Ghosh, Mehta and Khan, 2019) and characterize the diversity and abundance of microbial populations in biological specimens. Hence NGS technologies have emerged as popular tools for microbial profiling and pathogen detection (Tun *et al.*, 2012; Skarżyńska *et al.*, 2020; Grützke *et al.*, 2021).

Taxonomical profiling analysis in the metagenomics discipline uses two popular approaches: the 16S rRNA and the shotgun metagenomics sequencing-based approaches (Jovel *et al.*, 2016). The 16S rRNA sequencing-based method uses polymerase chain reaction (PCR) to amplify hypervariable regions of bacterial 16S rRNA gene and compares these regions to a 16S reference database (DB) (Johnson *et al.*, 2019). In contrast, the shotgun metagenomics sequencing-based approach sequences all given DNA present in a sample (Sharpton, 2014). Although lower in cost (Breitwieser, Lu and Salzberg, 2019), 16S rRNA markers are limited to genomes of bacteria and archaea, and are subject to amplification biases (Woese, Kandlert and Wheelis, 1990; Janda and Abbott, 2007). On the other hand, the taxonomical profiling using shotgun metagenomics sequence data compares the sequences to reference whole-genome database . Since the data contain all genetic information present in the sample, this approach avoids the amplification biases observed in 16S rRNA sequencing (Fouhy *et al.*, 2016; Ranjan *et al.*, 2016) and increases the resolution of microbial identification (Durazzi *et al.*, 2021). Most importantly, it has broader applications such as functional profiling and identification of viruses and other microorganisms with simple genomes (Clark and Pazdernik, 2016).

Currently developed shotgun metagenomics sequencing-based taxonomical profiling software can be separated into two groups: the alignment-based and the alignment-free software. Alignment-based software, including BLASTN(Altschul *et al.*, 1990; Johnson *et al.*, 2008; Camacho *et al.*, 2009), which aligns sequences at the nucleotide level, and Diamond (Buchfink, Xie and Huson, 2015), which aligns at the protein level, have high sensitivity and have been used as the standard for metagenomics profiling. However, these software require large amount of time and computational resources to build genome alignements for the high number of sequences usually involved in metagenomics profiling studies (Cannings, 2004; Zielezinski *et al.*, 2017). Furthermore, recent investigations in alignment-based methods have reported that alignment-based software decrease in sensitivity with the use of mosaic genomes (ex. viruses) (Zielezinski *et al.*, 2017). To overcome these limitations, multiple software have been developed using alignment-free algorithms. For example: 1) Kraken2 (Wood, Lu and Langmead, 2019, p. 2) and CLARK (Ounit *et al.*, 2015) were designed with k-mer matching algorithms, where only substrings of sequences were matched (Healy and Chambers, 2014); 2) Metaphlan3 (Truong *et al.*, 2015; Beghini *et al.*, 2021) was designed to identify unique genetic markers within each microbial taxon; and 3) Centrifuge (Kim *et al.*, 2016) and Kaiju (Menzel, Ng and Krogh, 2016) were designed to optimize the time and resources of profiling by compressing the reference microbial genomes into the index structures for storaging and searching (at the nucleotide and protein levels, respectively) (Burrows and Wheeler, 1994). In addition to the software mentioned above, some methods were developed to improve the results of existing software, such as Bracken (Lu *et al.*, 2017) that improves Kraken2’s output by eliminating false positive assignments using a Bayesian framework, and CLARK-s (Ounit and Lonardi, 2016) that improves the sensitivity of CLARK with the use of spaced Kmers. Previous benchmarks on shotgun metagenomic sequencing taxonomical profiling software have evaluated the performances of these software using either in silico or in vitro datasets (Peabody *et al.*, 2015; Escobar-Zepeda *et al.*, 2018; Ye *et al.*, 2019). The advantage of using these artificial datasets is that their performances can be evaluated by comparing their microbial profiles with the known composition of the artificial datasets. However, the performances of these software to analyze the microbial profiling and diagnostic applications in biological specimens has been less studied. For samples collected from wild animals, the microbiome compositions are unknown and potentially contain taxa that do not have genomes available in the reference DB. These situations can become a potential source of technical errors for accurate detection and profiling a sample's microbiome.

In this study, we compared the microbial profiles of tissue samples from two species of *Rattus* (*Rattus rattus* and *Rattus norvegicus*) using different metagenomic software and DBs. Specifically, we 1) compared the taxonomical profiles classified by four DBs and nine metagenomics profiling software listed above, 2) determined their effect in the downstream analyses and result interpretation; and 3) identified the presence of potential zoonotic pathogens such as *Leptospira* from each software’ profiling results.

**Materials and Methods**

**Samples.** Tissue samples from kidney (K), spleen (S), and lung (L) were obtained from four rats from two different species, *Rattus rattus* (R28) and *Rattus* *norvegicus* (R22, R26, and R27). Rats were captured from the island of Saint Kitts (longitude 17.3434° N and latitude – 62.7559°W) following protocols approved by the Ross University School of Veterinary Medicine (RUSVM) IACUC (approval # 17-01-04). DNA was extracted from samples using DNeasy Blood and Tissue Kits (QIAGEN Scientific Inc., MD, USA), following the manufacturer's protocol.

**Metagenomic shotgun sequencing.** DNA sample quality was assessed via analysis of the DNA purity and integrity with the agarose gel. DNA purity (OD260/OD280) and concentration were measured using the Nanodrop and Qubit 2.0. The library for metagenomic sequences was constructed with 1 μg DNA per sample. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina following manufacturer's instructions. The DNA sample was fragmented (350 bp), end-polished, A-tailed, ligated with Illumina sequencing adaptor and amplified with the PCR technique. The PCR products were then purified for sequencing. Before sequencing, samples were clustered on a cBot Cluster Generation System, then sequenced on an Illumina HiSeq platform for paired-end reads.

**Data pre-processing**

Sequencing adapters, low-quality reads, and host DNA reads within the metagenomic samples were removed using the software KneadData (The Huttenhower Lab, no date) with the default Trimmomatic (Bolger, Lohse and Usadel, 2014) (version 0.33) settings (SLIDINGWINDOW:4:20 MINLEN:50) and the "—very-sensitive" Bowtie (Langmead *et al.*, 2019) (version 2.3) option. The hosts' reference sequences, which were used to separate host reads from the microbial reads, were downloaded from the NCBI's RefSeq DB (Human: GCA\_000001405.28\_GRCh38.p13; *R. norvegicus*: GCF\_015227675.2\_mRatBN7.2; *R. rattus*: GCF\_011064425.1\_Rrattus\_CSIRO\_v1).

**Metagenomic profiling**

Software. Nine software (BLASTN, Diamond, Kraken2, Bracken, Centrifuge, CLARK, CLARK-s, Metaphlan3, and Kaiju) were chosen to determine the rats tissues' metagenomic profiles. All software were used with the default settings according to the instruction manuals provided by the developers.

Database building. If the software had pre-built DBs, these were downloaded directly from the software’ homepage (BLASTN, minikraken DB of Kraken2, Centrifuge, and Metaphlan3). Otherwise, DBs were built based on the standard instructions provided by the software’ manual (CLARK, CLARK-s, Diamond, and Kaiju), with the exception of software that had their DBs available online with the contribution of the scientific community. In this case, the DBs were downloaded directly from the online resources (standard DB of Kraken2, maxikraken DB of Kraken2, and Bracken). Detailed information about DB building is available in Table I.

For this specific analysis, a custom Kraken2 DB was built following the manual’s instructions. All the libraries present in Kraken2’s standard DB (which include NCBI RefSeq’s bacterial, archaeal, viral, human genome, and known vectors (UniVec\_Core) libraries) were included in the customized DB, with the addition of the genomes from the two rat species: *R. norvegicus* (GCF\_015227675.2\_mRatBN7.2) and *R. rattus* (GCF\_011064425.1\_Rrattus\_CSIRO\_v1).

**Statistical analysis**. Metagenomic profiles were loaded into R (R Core Team, 2020) for statistical analysis using the package “phyloseq” (McMurdie and Holmes, 2013). Pairwise significant difference assessments were performed by Wilcoxon signed-rank test implemented in R’s “rstatix” package (Kassambara, 2021), which is a non-parametric statistical hypothesis test used for comparing repeated measurements on a single sample. Alpha (Shannon, 1948; Simpson, 1949) and beta diversity (Bray and Curtis, 1957) indices (Whittaker, 1960) were used to describe the microbiome compositions within and between samples, respectively, and were calculated with the R package "vegan" (Oksanen *et al.*, 2013). The differentially abundant (DA) taxa analyzed between samples collected from two different tissues were determined by the R package "DeSeq2" (Love, Huber and Anders, 2014) using the "Wald” test, which normalizes reads classified under each species taxon with the “poscounts” method. The data visualization for the metagenomics profiles was performed using the R package "ggplot2" (Ginestet, 2011). For all statistical analysis, p-values were adjusted with the Holm-Bonferroni method (Holm, 1979). Results with p-adjusted value (padj) < 0.05 were identified as significant.

**Results**

**Computational Resources for DB Setup and Microbial Profiling**

To assess the outcome of the analysis using software/database combinations, four different Kraken2 databases (minikraken, standard, customized, and maxikraken) and nine different profiling software were used to classify the microbial communities of the wild rat samples. Details of the DBs used for each software in this study , as well as the computational resources and time used to build each software, are available in Table 1 and described in Supplementary Text1. Alignment-free software had to trade off the time and computational resources to build DBs for the speed of microbial profiling. Fortunately, most of them had pre-built DBs available online, which significantly decreased the time and resources required to use these software. For software without pre-built DBs available, CLARK and CLARK-s, required the most computational resources and time for DB building (Table I). Alignment-based software, BLASTN and Diamond, took the longest time for microbial profiling.

**Differences in Microbial Profiles Classified Using Different DBs and Software**

Significant differences were identified between the microbial profiles of the rat samples when different DBs were used (Table SI.1). The average number of total classified reads are shown in Table SI.2. The number of reads classified under the four highest taxonomy levels (Domain), Eukaryota, Bacteria, Viruses and Archaea, by each DB, are presented in Figure 1a-d. The padj values for all comparisons between DBs are available in Table SI.3. For the Eukaryota taxon, all but the number of reads classified by the standard and customized DBs were found significantly different (Figure 1a). For the Bacteria taxon, all the pairwise comparisons of the number of reads classified by the different DBs were found to be significantly different (Figure 1b). Classification results for Viruses are more similar across DBs than the results for Eukaryota and Bacteria. Only the comparisons between standard and customized DBs, and between minikraken and maxikraken DBs were not significantly different. In the case of Archaea, only the classification results of minikraken were found significanly different when compared with the results of other DBs, and the classification results of the other three DBs were not significantly different between each other.

To compare the profiling compositions of the different sofware, we calculated the number of total classified reads for each sample and determined these samples’ profiling compositions at multiple levels (Table SII.1). The average number of total reads classified ranges from n=10,955 using CLARK-s to n=77,499 using Diamond (Table SII.2). The number of unique taxa classified by each software varied considerably from 18 by Metaphlan3 to 4,816 taxa by Kaiju (Table II.S2). Furthermore, we found that Metaphlan3 did not classify any reads in samples of Rattus R26 (R26.K, R26.L. and R26.S) and sample R22.L and R27.K, while other software classified on average 1,252 (SD: 1408), 32,748 (SD: 32,178), 133 (SD: 112), 111,068 (SD: 113,203), and 4,011 (SD: 4,325) reads with these five samples, respectively (Table SII.2).

We classified reads by each software at the domain level taxa into Eukaryota, Bacteria, Viruses, and Archaea (Figure Ie-h, Table SII.3). All software except Centrifuge and Diamond showed the largest and significant differences across different software’s classification results for the number of reads classified into the Eukaryota taxon. Furthermore, due to the limitation of their DB compositions, Metaphlan3, CLARK-s, and Kaiju did not report reads classified into the Eukaryota taxon (Figure 1e). Compared to reads classified into the Eukaryota taxon, the number of reads classified into the Bacteria taxon were similar between software (Figure 1f). The reads classified by CLARK and CLARK-s were significantly different in bacterial composition when compared owith ther software included in this study. Regarding Bacterial composition, classifications by Metaphlan3 and Kaiju were the only two found similar with the ones by CLARK and CLARK’s (Table SII.3). The reads classified into Viruses by different software, on the other hand, were divided into two groups. The first group includes the classifications of BLASTN, CLARK, CLARK-s, Metaphlan3, and Kaiju, and the second group includes the classifications of Kraken2, Bracken, and Centrifuge. Classification results are not significantly different within each group, but different between groups (Figure 1g, Table SII.4). Diamond’s classification did not identify any reads as Viruses. Archaea’s read classification was very similar across software (Figure 1h, Table II.3), with the exception of Centrifuge being significantly different from most of other software (BLASTN, Diamond, Kraken2, CLARK, and CLARK-s). Bracken and Metaphlan3 did not classify any reads into the Archaea taxon (Figure 1h).

At the Phylum level, the number of unique taxa identified by each software ranged from 5 using Metaphlan3 to to 59 using Kaiju. We extracted the top 5 phylum taxa identified from each sample and combined reads classified to other phyla into the “p\_\_Other\_Phyla” taxon (Figure 2). The top 5 Phyla described a large percentage of read classification for all software. However, the distribution of reads classified into different phyla taxa are different across software. For example, Virus taxon, “p\_Pisuviricota”, contributed to over 85% (569/665) of the reads classified in sample R22.K using BLASTN (Figure 2a), but this taxon was not identified by any other software. Metaphlan3 classified all the reads in sample R22.K into “p\_\_Viruses\_unclassified” (Figure 2h), and CLARK and CLARK-s classified 63% (120/190) and 57% (95/166) of sample R22.K’s reads into two different Virus taxa, “p\_\_Uroviricota” and “p\_\_Artverviricota” (Figure 2f-g). Kaiju also classified 21% of sample R22.K’s reads into “p\_\_Artverviricota” (34/157) (Figure 2i). Similar read distributions involving Virus classification were observed in samples R26.K, R26.S, and R27.K, where BLASTN classified 54% (657/1207), 20% (28/140), and 11% (422/3794) of reads into “p\_Pisuviricota” (Figure 2a), respectively; CLARK and CLARK-s classified a large percentage of reads into Virus taxon “p\_\_Uroviricota” (CLARK: 71% (636/900), 31/76 (41%), and 18% (201/1099); CLARK-s: 18% (50/271), 18% (7/67), 10% (83/1334), respectively) (Figure 2f-g), but other software only identified zero or a small number of reads into a Virus taxon in these samples (Kraken2 classified four reads into taxon “p\_\_Uroviricota”, Figure 2c). The distribution of Bacteria reads classified by BLASTN, Kraken2, Bracken, Centrifuge, CLARK, CLARK-s, and Kaiju are relatively consistent across samples. The diversity of taxa identified by Metaphlan3 is significantly lower than the ones identified by other software (Figure 2h). For example, Metaphlan3 identified 100% of sample R27.L’s reads as “p\_\_Proteobacteria, while other software identified 29% (SD: 12%) of R27.L’s reads as “p\_\_Proteobacteria” on average, with the unique number of Phylum taxa identified ranging from 2 by Diamond (91% of reads classified as “p\_\_Tenericutes”) to 50 by Kaiju. Diamond’s classification also showed differences in read classification when compared to other software (Figure 2b). The most notiable difference was the relative abudance of taxon “p\_\_Firmicutes” across samples. In the lung samples, “p\_\_Firmicutes” was classified on average in 17% of R22.L (SD: 9%), 20% of R26.L (SD: 9%), and 14% of R27.L (SD: 8%) by other software, but Diamond only classified 2% (133/4900) of reads as “p\_\_Firmicutes” in sample R26.L, and 0% in both R22.L and R27.L. On the other hand, Diamond identified a relative larger proportion of reads as “p\_\_Firmicutes” in samples R27.S (24%) and R28.L (19%) compared to other software (R27.S: mean: 2%, SD: 2%; R28.L: mean: 3%, SD: 3%), except for Centrifuge (R27.S: 24%, R28.L: 9%).

For the species level classification, the number of reads classified under taxa (strains) with the same species was aggregated to obtain a unique number of species classified by each software (Table SII.1). Out of all software, Metaphlan3 classified the least number of species taxa, with only 18 species (Table SII.4) while Kaiju classified the most, 4128 species (Table SII.4). From the species level classifications, 9 species taxa were identified by all nine software (*Leptospira interrogans*, *Leptospira borgpetersenii*, *Faecalibacterium prausnitzii*, *Bordetella pseudohinzii*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Bacteroides uniformis*, *Phocaeicola vulgatus*, and *Bartonella elizabethae*) (Table SII.1). Centrifuge vs Kaiju had the largest overlap of number of identified species taxa (2285), followed by Kraken2 vs Centrifuge (1737) and vs. Kaiju (1723) (Table SII.4). The species-level classification of the three software mentioned above shared a total of 1379 species taxa. In addition, BLASTN shared 1253 species taxa with Centrifuge, 1207 with Kaiju, and 1126 with Kraken2. CLARK and CLARK-s’ classification shared 1219 and 1059 species taxa wtith Kaiju, respectively. To assess if different software had identified the same species taxa as the most abundant taxa, species taxa with at least 10% of the reads from each sample were selected from each software’ classification. Metaphlan3 identified most of the number of unique species taxa (18), while BLASTN and Kaiju identified the least (7). CLARK vs. CLARK-s and Kraken vs. Bracken shared most of the number of taxa in this category (9 and 8, respectively). Two species taxa were identified by all software as the top ten percent most abundant species taxa, which were *L. interrogans* and *Bartonella elizabethae* (Table SII.1).

**Downstream analyses for microbial community characterization**

*Within-sample diversity (α-diversity)*

To understand how differences in the classification results can directly impact the the microbial communities characterization in each sample, we calculated two alpha indices (Shannon and Simpson) at the species level. Then, we characterized both the species richness (diversity) and evenness (abundance) of the microbial communities within each *Rattus* sample and compared them with the observed unique number of species identified by each DB and software (Observed).

The observed unique taxa classification results across all four DBs were significanly different from each other (Figure 3a). For species richness characterization within a community using the Shannon indices, only the indices obtained from minikraken DB were significantly different from the results obtained with the other DBs (Figure 3b, Table SI.4). Moreover, the Simpson indices, which weigh the microbial community’s characterization with the proportion of species within each sample, were mostly similar between the results of the four DBs (Figure 3c, Table SI.4). Only the Simpson indices obtained from the results of the standard and customized DBs comparison were significantly different (Figure 3c, Table SI.4).

The number of unique observed taxa (Table SII.5, Figure 3d) across different software were largely divergent from each other. Out of the 36 pairwise comparisons between different software, only 6 comparisons were not significantly different (Table SII.5), which were BLASTN’s observed taxa with Kraken2, CLARK, and CLARK-s, comparison between CLARK and CLARK-s, and comparison between Centrifuge and Kaiju. The Shannon indices showed more similarity between software than the unique observed taxa, however, they still had 23 out of 36 comparisons between software significantly different from each other (Table SII.5, Figure 3e). The Simpson indices were least impacted by the differences in classification results across software. Only 7 out of 36 comparison were found to be significantly different (Table SII.5, Figure 3f). Most of these were identified in comparisons between CLARK-s (3/7) and Centrifuge (4/7) with other software. The Simpson index between CLARK-s and Centrifuge’s classifications were also significantly different between each other.

*Between-sample diversity (β-diversity)*

The pairwise relationships between every two *Rattus* samples were determined with the Bray-Curtis (BC) dissimilarity index, and clustered hierarchically. The BC indices were found to be significantly different across all DBs, except for those reported by the results of maxikraken and customized DBs (Table SII.6). The hierarchical clustering analysis also shows dissimilarities across results when using different DBs (Figure 4a). Three kidney samples (R22.K, R26.K, and R27.K) were found to be clustered with one of the spleen sample (R26.S) in all four DBs’ classifications. However, their relationships with another spleen sample (R27.S) changes with the type of DB used. Despite the differences in the more granular hierarchical clusters, the two major clusters describing the general relationships between samples did not change with the use of different DBs. Three lung samples (R22.L, R26.L, and R27.L) always clustered closely together away from the other samples, while all kidney and spleen samples formed a separate cluster with the other lung sample (R28.L).

The hierarchical clusters describing the general relationships between samples remained consistent across all different software (Figure 4b). Except for Metaphlan3, all the other software aggregated the *Rattus* samples into two large clusters: the first with three lung samples (R22.L, R26.L and R27.L) and the second with a combination of all the kidney and spleen samples, and one lung sample (R28.L). When comparing the BC indices reported by different software (Table SII.6), we found that BLASTN’s BC indices were more similar than the ones of Kraken2, Bracken, and Centrifuge, while the BC indices of CLARK and CLARK-s were more similar with those reported by Diamond, Kaiju, and Kraken2. Metaphlan3, with 5 out of 12 samples unclassified, was significantly different from the other software (Table SII.6).

*Differentially abundant (DA) taxa identification*

DA taxa between samples of different tissues were identified to show the most significantly different microbial taxa between the microbiome of two tissues. For DA taxa identified from lung versus kidney samples at the species level, the number of DA taxon identified by the use of different software ranged from 10 (Diamond) to 596 (Centrifuge) (Table SII.7, Figure 5a). The abundance was significantly higher in in the kidney than in the lung samples for all software’ classifications (Figure 5b). Five significantly abundant species (*Bordetella pseudohinzii*, *Bordetella bronchiseptica*, *Leptospira interrogans*, *Leptospira borgpeterseni*, and *Mycoplasm pulmonis*) were classified by all software (Table SII.7). Kaiju and Centrifuge had the highest number of distinct DA taxa (390 and 376 taxa, respectively) (Figure 5a). Although Centrifuge identified the largest number of DA species taxa, Kaiju identified the highest number of unique phylum taxa (42), which means that many of Centrifuge’s DA species have the same phylum taxonomy (Figure 5a).

To obtain a more generalized overview of the DA taxa identified from the classification of each software, we aggregated the lung versus kidney DA species taxa into the phylum level and visualized the presence and absence of each phylum taxon as the DA taxa across the different software (Figure 5c). At the Phylum level analysis, the results were consistent for all software except for Diamond, which missed four taxa ("p\_\_Aquificae”, “p\_\_Fusobacteria”, “p\_\_Firmicutes”, and “p\_\_Cyanobacteria”). Kaiju and Centrifuge were the only two software that reported virus taxa (“p\_\_Negarnaviricota”, and Kaiju reported “p\_\_Nucleocytoviricota” and “p\_\_Uroviricota”) as DA. Archaea taxa were only reported by Kaiju, Centrifuge, and BLASTN. All three software reported "p\_\_Euryarchaeota”, and both Kaiju and Centrifuge reported “p\_\_Candidatus Micrarchaeota” and "p\_\_Candidatus Lokiarchaeota”. Finally, Kaiju uniquely reported “p\_\_Candidatus Thermoplasmatota”.

DA taxa were also identified between the microbiomes of lung and spleen and between kidney and spleen samples. Kaiju and Centrifuge were the most sensitive in DA identification between lung and spleen samples, but the sensitivity as similar across software when the differences between microbiomes of two tissues are relatively similar (kidney vs. spleen). Diamond was the least sensitive in the DA analysis across all three comparisons between tissues. The detailed comparisons are described in supplementary Text2.

**Pathogen detection**

We focused on *Leptospira* detection since rodents are the major reservoirs of this major zoonotic pathogen. We identified *Leptospira* in the three tissues of all four subjects by the nine software, however, each software reported *Leptospira* in different samples (Table II). Centrifuge was the only one that reported *Leptospira* in all of the 12 Rattus samples, where 9 unique *Leptospira* species were identified (8 from the pathogenic group and 1 from the saprophytic group) (Table SIII.1). Kaiju also identified *Leptospira* from 9 out of 12 samples with 8 unique species (7 from the pathogenic group and 1 from the saprophytic group) (Table SIII.1). Kraken2, following Centrifuge and Kaiju, classified 6 *Leptospira* in 6 samples with 3 unique species all from the pathogenic group (Table SIII.1). Except for Metaphlan3, all software identified *Leptospira* from two of the kidney samples (R22.K and R28.K), which have on average 31 (SD: 3) and 84,344 (SD: 2.2) reads classified under *Leptospira* (Table SIII.2), respectively. BLASTN, Centrifuge, Kaiju, Kraken2 and CLARK identified *Leptospira* from a lung sample (R22.L). Metaphlan3 only identified *Leptospira* in one of the kidney samples (R28.K). All samples identified by at least three software had at least a total of 30 reads classified under *Leptospira* (Table SIII.2). Samples that were only identified by Kaiju or Centrifuge had on average only 2 (R27.K, SD: 1) to 15 (R26.L, SD: 2) reads classified under *Leptospira* (Table SIII.2). *Leptospira* detection was also dissimilar when different databases were used for Kraken2’s classification (Table SIV). Kraken2’s analyses with the maxikraken DB identified *Leptospira* in all samples, while standard and customized DBs identified *Leptospira* in two lung samples (R22.L and R27.L). Standard DB also identified *Leptospira* in the three spleen samples (R22.S, R27.S and R28.S). *Leptospira* detection in the kidney samples using three traditional methods (PCR/DFA/Culture), in comparison to the metagenomics data is shown in Table II.

**Discussion**

The field of metagenomics, developed with the advancement of NGS technologies, allows scientists to build a complete and discriminatory microbial profile with virus, archaea, and bacteria taxa for samples collected from the environments of interest (Jovel *et al.*, 2016). These metagenomic profiles can also be used to detect relevant pathogens in clinical and epidemiological investigations (Qin *et al.*, 2012; Knights, Lassen and Xavier, 2013) and to observe the interactions between micro-ecosystems and their changing environments (Handley, 2019). Researchers achieve this using a number of bioinformatic analysis software and database combinations. In this study, we identified differences in the microbial profiles when different metagenomics classification software were used. Our results show that there are in fact differences in the classification outputs when different taxonomical profiling software on the same dataset are used. We conclude that the selection of methods influences the results of microbiome characterization, and potentially could lead to different biological conclusions and misinterpretation of pathogen presence.

Our study uses real life samples and is different from previous benchmarking studies (Escobar-Zepeda *et al.*, 2018; Ye *et al.*, 2019) which are based on *in silico* datasets or with the support of laboratory synthetic samples. Our study provides the evidence of reporting false positive or false negative taxonomies if appropriate DBs, or software are not used. Although these differences seem negligible in the benchmarking studies for tools with similar algorithms, they can lead to diverging biological conclusions in the downstream analyses depending on the questions being asked. The biases reported have been understudied; and therefore, it is crucial to demonstrate the effect of these biases with real biological data, to raise awareness and identify the potential factors that lead to incorrect biological conclusions in a metagenomics study.

**Biases introduced by DB selection**

Incorrect taxonomical profiling of the collected samples’ microbial community can start from choosing an incorrect DB. All current taxonomical profiling software require a large number of computational resources for DB building and storage. Some software, such as Kraken2, provide an alternative pre-built DB for users with inefficient computing resources, which minimize the size of the DB to be loaded into a machine with RAM as low as 8 GB. There are also multiple versions of Kraken2’s DBs provided by the science community that can be easily downloaded and updated frequently. For example, the Langmead lab builds the most recent version of Kraken2’s standard DB based on NCBI’s RefSeq library routinely. In addition, the Loman lab has built a Kraken2 DB with the inclusion of draft genomes that were not included in the Refseq library. Both of these two Kraken2 DBs are freely available online, and minimize the workload of building a database from scratch. However, all three DBs mentioned above included the human genome as the only Eukaryotic genome in the DB, which is not the host of our dataset. The biases introduced from host genomes included in the DB for metagenomics analysis have been addressed previously (Pereira-Marques *et al.*, 2019). Building a separate DB (customized database for our dataset) with the inclusion of the two Rattus hosts genomes on top of the standard DB corrected this issue. We found that although the number of reads classified using different DBs differ significantly from each other, the characterization of each sample’s microbial communities was not largely biased by the use of different DBs. In our analyses, only the richnesses of the samples (Shannon indices), which accounts for the rare species within the community obtained from the miniKraken DB, were significantly different when compared to the richness characterized by other DBs. On the other hand, the eveness of each sample’s microbial community measured with the Simpson indices was mostly consistent across the classifications with different DBs. For microbial communities between samples, we found that only the clusters describing the most distinctive relationships between samples were consistent across the classifications of all DBs. Sophisticated relationships between samples were altered by the biases introduced from DB selection.

**Resources required to use different software**

Metagenomics software can be classified into two different categories, alignment-based and alignment-free. The alignment based software, which suffers greatly from slow speed and the need of large resources, are generally thought to have high sensitivity. On the other hand, the alignment-free software utilize relatively small computational resources and significant improvement in speed of the analysis.In our study, the two alignment-based software, BLASTN and Diamond, were the two most time intensive software. They took two and five hours, respectively, on average to complete the analysis for one sample, while other software took at most three minutes for the same task. The time and resources required to build the DBs for the alignment-free software became the trade-off for the speed of the analysis itself. For example, the building of CLARK’s DB took almost two days with 400 GBs of memory used. Fortunately, most of the software included in our study have pre-built DBs distributed with the release of the software (except for CLARK, CLARK-s, Diamond, and Kaiju). However, if the analysis requires the identification of taxa that are not included in these pre-builit DBs, the time and resources added to the metagenomics profiling analysis will increase significantly.

To compare the metagenomics profiles classified by each software, we chose the standard DBs provided by the developers of these software. If the standard DBs were not indicated, we built the DBs with the genomes of Bacteria, Archaea, Viruses, and Human available in NCBI’s RefSeq library, which are the compositions for most of the pre-built DBs. CLARK-s’ DB was required to be built on top of a CLARK DB of the same composition, but when the DB was built with the genomes of Bacteria, Archaea, Viruses, and Human, the building was suspended by the software with the error message “the number of targets exceeds the limit (16383)”. This limitation was reported to the developer of CLARK-s, but it has not been resolved by the time this manuscript was drafted. We bypassed the limitation by building the DB with Bacteria, Archaea, and Viruses genomes separately, and combining the classifications using each DB at end of the analysis. In addition, Metaphlan3, which identifies the microbial taxon with marker genes, does not have an option to build a customized DB, only the marker DB distributed by the developer could be used for profiling.

**Biases in microbial profiles introduced from software selection**

At the Domain level, Eukaryota taxon has contributed the most to the dissmimilarities between the different software classifications. Almost all pairwise comparisons between the Eukaryota profiles classified by each software were found significantly different between each other. Compared to the number of reads classified under Eukaryota, the number of reads classified under Bacteria, Viruses and Archaea taxa by different software were much more similar. The classifications of Centrifuge, CLARK, and CLARK-s were frequently identified significantly different from the ones of other software regarding the number of reads mapped to Bacteria and Archaea. The classifications of Viruses, on the other hand, were found separated into two groups where the classifications within a group were similar (group1: BLASTN, CLARK, CLARK-s, Metaphlan3 and Kaiju; group2: Kraken2, Bracken and Centrifuge). Diamond did not identify any reads as viruses. This division in virus classifications was further validated by the virus classifications at the lower taxonomy levels. The samples with large percentage of reads classified under virus taxa by group1 software were not profiled by software in group2. Although software in group1 were more sensitive in virus identification than of group2 software, the exact virus taxa and the correspondent number of reads using different group1 software were not consistent. The virus taxon identified by BLASTN in high abundance was not identified by any other software included in the analysis. Except for the samples with virus classifications, the profiling of Bacteria taxa was found mostly consistent across the software at both Phylum and Genus level. Only the classifications of Metaphlan3, which could only identify a few taxa from each sample with high abundance, and Diamond, which reported conflicting profiles in Firmicutes identification at the Phylum level (Bacillus at Genus level) with the classification of all the other software, were different from other software in Bacteria classification.

Compared to Phylum and Genus levels, the classifications at the species level were more divergent across software. Although most software reported more than 1,000 unique species taxa from the Rattus profiles (except for Bracken and Metaphlan3), only nine were identified by all software, and only 2 species were found overlapping in taxa with at least 10% in relative abundance.

**Microbial community characterization**

In addition to the differences in microbial profiles classified by different software, the differences across the richness of each samples’ microbial community were significant in the majority of the comparisons across software. Most of these were found between the classifications of Kraken2, Metaphlan3, Centrifuge, and Kaiju with other software. However, when richness was measured with species abundance (Simpson index), the characterizations were mostly not affected by use of different software. The characterizations of the relationships between-samples were divergent across software, but the most discriminatory relationships within the rat samples (between lung and other samples) were captured by most of the software (except for Metaphlan3).

**Differences in differential abundant(DA) taxa**

In order to address potential biases introduced from software selection with biological significance, we identified the DA taxa between samples of different tissues in a pairwise fashion. From the between-sample relationship analyses, all software reported that the microbial communities of lung samples were distinct from the kidney and spleen ones. The classifications of all DA taxa reported at the species level were largely different across software. The largest range in the number of DA taxa reported by different software was found in the analysis between lung and kidney samples. Despite the large differences in the number of taxa identified, there was still a small number of overlapping species identified across the results of all software. We also found similarities in the software-overlapped DA taxa between lung vs kidney and lung vs. spleen analyses, where two *Bordetella* species and a *Mycoplasm* species were reported by all software in both analyses. More DA identified were overlapped across software at the Phylum level. In addition to the overlapped DA taxa, Kaiju and Centrifuge were more likely to report more taxa as differentially abundant than the other software. These two software were also the only two that reported both viruses and archaea taxa as DA (BLASTN only reported Archaea and CLARK only reported viruses). Diamond was found to be the least sensitive in the differentially abundant analyses for all three comparisons between tissue samples, where phylum taxa identified by all the other software were frequently not identified by Diamond.

To assess the sensitivity of shotgun metagenomics as a tool for pathogen diagnosis, we identified the presence of the zoonotic pathogen *Leptospira* in all of our tissue samples. Centrifuge and Kaiju were found the most sensitive software in diagnosing *Leptospira*, where Centrifuge reported the presence of *Leptospria* in all the samples. Except for Centrifuge and Kaiju, the rest of the software were consistent in *Leptospira* identification in only two of the kidney samples (R22.K and R28.K) and in one lung sample (R22.L). Since *Leptospira* colonizes the kidney of rats (Adler and de la Peña Moctezuma, 2015), we compared the results from three traditional methods (PCR/DFA/Culture) applied to kidney samples reported in a . We found that most software included in our analysis had similar sensitivity in *Leptospira* identification with traditional methods, except for PCR. In addition, Centrifuge reported the presence of *Leptospira* in samples that were not reported by any other software or a traditional method. This identification could be due to Centrifuge’s better performance in sensitivity, or as a result of false positive reporting. Furthermore, we found that Kraken2 with maxikraken DB also reported *Leptospira*’s presence in all samples.

The inconsistencies found between the results of different metagenomic software showed that significant biological conclusions from metagenomic profiling analyses have the potential to be only the artifacts of the software’ algorithms. Shotgun metagenomics sequences might be too short for current taxonomical profiling software to differentiate microbial taxonomies between similar genomes (Tran and Phan, 2020). The use of real-world datasets has the advantage of addressing this challenge, reminding the investigators to stay skeptical with the classification results obtained from the profiling software. On the other hand, benchmarking the software’ performances with the real-world dataset, in contrast to using *in silic*o datasets, has the limitation of lacking knowledge about the true microbial compositions within each sample, which means we could not evaluate the performance of software based on their degrees of accuracy and sensitivity, nor giving direct suggestions on software’ selection. In addition, metagenomics profiling has been broadly utilized in many fields of studies, including clinical, pharmaceutical, as well as ecological. Each field uses microbial profiles differently based on the biological question proposed. Our choice of the real-world dataset could only address a limited number of software selection biases. We suggest researchers from different study fields to be aware of the possible error-prone conclusions made from metagenomics profiling analysis, and evaluate it objectively comparing it to other traditional methods (e.g. PCR, culture, or serotyping).

Advancement in sequencing as well as computational technologies allows modern-day biological research to move to a brand-new era. However, while benefiting from the powerfulness and convenience of technologies, we should always critically analyze and validate software outputs based on our prior knowledge and available evidences .

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**Conflicts of interest**

No conflict of interest declared.

**Repositories**

The raw sequence files (FASTQ) were submitted to the NCBI Sequence Read Archive under the Bioproject accession number: PRJNA717669. The individual isolates can be accessed under the following Biosample accession numbers: SAMN18507082 - SAMN18507091. All scripts for this publication are freely available on the following Github link: <https://github.com/salvadorlab/MetagenomicsToolsEvaluation>.

**Data summary**

The raw sequence files (FASTQ) were submitted to the NCBI Sequence Read Archive under the Bioproject accession number: PRJNA717669. The individual isolates can be accessed under the following Biosample accession numbers: SAMN18507082 - SAMN18507091. The short-read archive accession numbers are listed in Table S1.

**Ethical Approval**

Rats were captured following protocols approved by the Ross University School of Veterinary Medicine (RUSVM) IACUC (approval # 17-01-04).

**Supporting Information**

SI.kraken2\_db\_comparison.xlsx

SII.software\_comparison\_full.xlsx

SIII.lepto.diagnostic.xlsx

SIV.lepto.kraken2.db.diagnostic.xlsx

Supplementary figures file

References

**Figure and Table Legends**



**Figure 1.** Domain level microbial profiles for rat tissue samples using different DBs (a-d) and software (e-h). All pairwise statistical comparisons between profiles classified by different DBs and software within each domain were performed with a Wilcoxon signed-rank test with p-adj value available in Table SI.3 and Table SII.3 for DBs and software comparison, respectively. Samples: R22.K () , R26.K (), R27.K (), R28.K () ,R22.L (), R26.L (), R27.L (), R28.L (), R22.S (), R26.S (), R27.S ( ), R28.S (). **Figure 2.** Phylum level microbial profiles for rat tissue samples using nine different software (a-i). Each row panel represent microbial profiles classified using a different software. Left panel is the microbial profiles reported in absolute number of reads and right panel is the microbial profiles reported in relative number of reads. Phylums: P\_\_Other.Phyla (), P\_\_Pisuviricota (), P\_\_Proteobacteria () ,P\_\_Spirochaetes (), P\_\_Actinobacteria (), P\_\_Peploviricota (), P\_\_Tenericutes (), P\_\_Firmicutes (),P\_\_Bacteroidetes (), P\_\_Cyanobacteria (), P\_\_Cossaviricota (), P\_\_Acidobacteria (), P\_\_Aquificae (), P\_\_Artverviricota (), P\_\_Euryarchaeota (), P\_\_Uroviricota (), P\_\_Crenarchaeota (), P\_\_Candidatus (), P\_\_Chlamydiae (), P\_\_Fusobacteria (), P\_\_Preplasmiviricota (), P\_\_Viruses\_unclassified ().

**Figure 3.** The alpha diversity of the rat samples characterized by different DBs (a-c) and softwares (d-f) is described by the Observed, Shannon, and Simpson indices, which characterize each sample’s microbial composition based on diversity richness and abundance. All three indices were calculated based on the absolute number of microbial reads at the species level. All pairwise statistical comparisons between DBs and software within this figure were performed with a Wilcoxon signed-rank test with p-adj value available in Table SI.4 and Table SII.5 for DBs and software comparison, respectively. Samples: R22.K () , R26.K (), R27.K (), R28.K () ,R22.L (), R26.L (), R27.L (), R28.L (), R22.S (), R26.S (), R27.S ( ), R28.S ().

**Figure 4** Between-sample microbial composition dissimilarity measure by BC indices using different DBs (a) and software (b). These BC indices were characterized based on the number of microbial reads classified at the species level. Higher BC values indicate a high level of dissimilarity between the two samples’ microbial composition. The red () and yellow () colors show low (0) and high (1) levels of dissimilarity, respectively. Hierarchical clustering was used to cluster together samples that have similar microbial compositions (dendrograms on the left and top of the heatmaps). Pairwise statistical comparisons between DBs and software within this figure were performed with a Wilcoxon signed-rank test with p-adj value available in Table SI.5 and Table SII.6 for DBs and software comparison, respectively.

**Figure 5.** DA microbial taxa identified from comparing the microbial profiles of all rats’ lung and kidney tissues. The number of DA species and phylum identified using different software’s profile is shown at the bar plot directly left to the software names in a). The intersection between DA taxa identified by different software at the species level is shown at the barplot at the top of a), where the number of DA viruses taxa were colored in gray (). Dotplot at the bottom shown the combinations of intersections between software. Number of DA taxa at phylum and genus level is shown in b), where numbers of taxa significantly higher in abundance the kidney samples are colored in blue (), and numbers of taxa higher in the lung samples are colored in green (). The DA phylum taxa identified by each software is shown in c), where red (****)indicate the phylum taxa at each row is reported as differentially abundant by the classification of the software in every column, and blue (****) is not reported as DA taxa by the software. Each phylum taxa were also annotated by their corresponding Domain taxa, where dark blue is Bacteria (), yellow is Archaea (****), and Orange is Virsues ().