Towards complete representation of bacterial contents in metagenomic samples

Xiaowen Feng\textsuperscript{1,2} and Heng Li\textsuperscript{1,2}\textsuperscript{*}

\textsuperscript{1}Correspondence: hli@dfci.harvard.edu
\textsuperscript{2} Department of Data Sciences, Dana-Farber Cancer Institute, Boston, US
\textsuperscript{2} Department of Biomedical Informatics, Harvard Medical School, Boston, US

Full list of author information is available at the end of the article

Abstract

Background: In the metagenome assembly of a microbiome community, we may think abundant species would be easier to assemble due to their deeper coverage. However, this conjecture is rarely tested. We often do not know how many abundant species we are missing and do not have an approach to recover these species.

Results: Here we proposed k-mer based and 16S RNA based methods to measure the completeness of metagenome assembly. We showed that even with PacBio High-Fidelity (HiFi) reads, abundant species are often not assembled as high strain diversity may lead to fragmented contigs. We developed a novel algorithm to recover abundant metagenome-assembled genomes (MAGs) by identifying circular assembly subgraphs. Our algorithm is reference-free and complement to standard metagenome binning. Evaluated on 14 real datasets, it rescued many abundant species that would be missing with existing methods.

Conclusions: Our work stresses the importance of metagenome completeness which is often overlooked before. Our algorithm generates more circular MAGs and moves a step closer to the complete representation of microbiome communities.

Keywords: Metagenome, Binning, Metagenome-assembled genomes, assembly completeness

Background

De novo metagenome assembly has been an allusive promise of unbiased and comprehensive snapshot of microbial communities of interest, independent to isolation and cultivation \cite{1, 2, 3}. Neither of the two aspects has been close to realization. First, most past metagenome sequencing projects were based on short read sequencing and produced short contigs of tens of kilobases (kb) long, which need to be clustered to form metagenome-assembled genomes (MAGs). Most de novo genome-complete \cite{4, 5} MAGs still contain an average of 87 assembly gaps with median length about 1.3kb. We manually checked some and found that these gaps either have no presence in the BLAST nr/nt database using BLASTn, or were homologous to shared genes such as ribosomal RNA (rRNA) operons. Second, MAGs are rarely checked for their representation-completeness. Studies often assumed that sufficiently abundant or the most abundant species will be reconstructed \cite{6, 7, 8}.
There is no such guarantee despite efforts to approve it [9]. A distinct species with low coverage can be easier to recover than abundant but highly similar species or strains. Horizontal gene transfer (HGT) or large duplication events are even more difficult to resolve. One major obstacle for improving the situation was that 16S rRNA sequences, which is a proxy of species definition [10, 11, 12], were no easier to assemble than the whole genome [13] and will require amplicon sequencing [14, 15, 16]. Therefore it is hard to cross validate between gene-based composition inference and assembly [8, 17, 18].

Pooling short read MAGs to form a complete reference catalogs [19, 18, 20] is also flawed. The human gut microbiome is the most genome-sequenced metagenome context. SRA has near 83k results assigned to this category as of July 2022. However, recent large-scale studies still found 42% of their quality MAGs missing from major public repositories [18]. For gut microbiome of other species, this number can be as high as 86% [21]. Moreover, taxonomic profiling of 1004 faecal samples from TwinsUK registry found each species was observed in a median of 2.7% samples, with 12% of species being sample-specific and 50% species found in less than 1% samples [22], demonstrating vast diversities. To make the situation more complicated, there is no consensus on the optimal library size. Most sequencing projects prioritized sample size over high-coverage, perhaps due to the observation that MAG yield per gigabases sequenced per sample peaks at the lower end of library sizes (Figure S1-2). Some of the most deeply sequenced [23, 24] or unusual [25] datasets were analyzed unassembled. The endgame of the reference MAG accumulation process is unclear. On the other hand, the drawbacks of short read MAGs are still relevant even if MAGs are just treated as bags of genes [26, 27], or used for composition inference [11, 28].

Metagenome assembly using accurate long reads, such as Pacbio HiFi reads, can now recover haplotype-resolved near-complete closed MAGs and separate species with sequence divergence as low as 1% [29, 30, 31]. This somewhat resolves the first issue of short read MAGs, although the quality evaluation remains reference-dependent. Binning re-emerged to be problematic. Traditional binners tend to collected contigs from multiple haplotypes, despite they are well capable of creating high quality short read MAGs containing tens of hundreds of short contigs. In HiFi assemblies, only when the binner pulls less than ten contigs would a bin likely to pass quality checks, basically relying on the recruitment of a few long contigs and nothing more. The second issue about representation-completeness is an open question. However, with circular contigs no longer relying on binning and full length 16S rRNA sequences readily available from the HiFi reads, we think it is possible to approach it from both k-mer spectrum, learning from evaluations in single sample large genome assemblies, and 16S-based species-level operational taxonomic units (OTUs). It is no less important about learning what is absent from the assemblies than examining what has been recovered in order to properly compare across samples and different microbiotas, and further, to improve the assembly method.

This manuscript has three major components. We first introduce a graph topology-based, reference-free postprocessing step for the hifiasm-meta [31] assembler. The core is a circle-finding depth-first search. Although simple, this could replace about half of regularly binned MAGs with pseudo circular paths of identical or better quality, as well as adding in MAGs that are not recoverable by regular
binners. There is no external data needed, and the end results were very close to the almost-best-possible MAG recovery (i.e. if assisted by checkM). We then propose two evaluations as mentioned above and apply them to the HiFi MAGs. We demonstrate that in the best cases, near-complete MAGs alone could offer proper sample representations comparable to that of a comprehensive reference catalog and delegate almost 90% abundant OTUs (more than 30 16S copies, using 99% boundary).

Results

Topology-based cycle-finding on the contig graph as a binning method and its merging with traditional binning

Established binners were developed for binning short read metagenome assemblies. The process relies on tetranucleotide profiles and coverage estimates [32, 33], and sometimes consults assembly graph for further refinements [34]. Single-copy marker genes and other prior knowledge can also be exploited [35, 36], though such binning complicates the final evaluations, as assessing assemblies of empirical datasets heavily relies on checkM [4] or similar methods that also utilize marker gene sets and phylogenetic placement.

In short read assemblies, the binners are capable of pooling hundreds of short contigs to form very high quality MAGs. However, they do not perform as good in HiFi assemblies. We found most near-complete or high-quality MAGs generated by binners contain less than 10 contigs. The completeness of these bins mostly come from only one or two long linear contig(s). While this does not defeat the purpose of binning on HiFi assemblies, haplotypes that do not assemble well would remain missing. Additionally, long contigs of hundreds or more kilobases from closely related haplotypes are prone to be clustered together, which results in complete but highly contaminated bins. The first issue was especially hard to be improved. The second can be somewhat mitigated by arbitrarily splitting bins based on intra-bin sequence similarity. We noticed that the assembly graph of hifiasm-meta was simple enough to provide binning hints when the assembly was visually [37] reasonably not bad (Figure 1). We propose a simple topology-based assembly postprocessing in complement to binning to achieve a reasonable complete representation of the library. The method also has bonus contig ordering and circularization. We implemented this approach in the hifiasm-meta assembler. The other two capable assemblers, metaFlye and HiCanu, produces more tangled graphs or does not produce a graph, respectively.

We use two assumptions: a bacterial chromosome is expected to be circular, and its length to fall into the range of 1Mb-8Mb. We work on the primary assembly graph. We first collect cycles of roughly 1Mb-10Mb long on the contig graph via depth-first search (DFS). To balance redundancy and the risk of missing promising cycles, the DFS always try to use a contig that has not been a node in any of the collected cycles. The criteria are permissive and only terminates the search if 100-200 nodes have been used. If a search extends too long, we halve the stack and resume from halfway, in order to avoid missing cycles due to the arbitrary ordering of contigs.

We then remove duplicated cycles using Mash distance [38]. Mash distance correlates well with ANI in the range of 90-100% and we use bottom 1000 hashes with
**Figure 1.** (a) Sankey plot showing flow of reads and contigs of sheep-gut-1b. Left: reads to contig mappability ("minimap2 -cxmap-hifi"). Middle: contig binning categories. Right: MAGs quality categories. Group heights normalized by counts, except the left-most side which is normalized by base pairs. The heights of “not aligned” and the “dismissed” have no meaning. (b) Bandage [37] plot of sheep-gut-1b primary assembly: (top-left) primary assembly graph, (top-right) circular path candidates to rescue marked in blue, (bottom-right) high-quality-or-above rescued circles marked in green, and (bottom-left) best possible high-quality-or-above MAGs marked in red. Note that the size of circle-shaped subgraph that consists of numerous short contigs was exaggerated by the graph layout method.
k-mer size of 21 per recommendation in the Mash paper. If the two cycles being compared differ in length for more than 1Mb, we do not drop any of them regardless of Mash distance. We set the species boundary at 95% whole-genome Average Nucleotide Identity (ANI), which was derived from DNA-DNA hybridization values and later reconfirmed by large-scale studies [39, 40]. We also tried 97% as a slightly relaxed threshold, and 99% which was the highest possible ANI for most pairs of long circular contigs produced in HiFi assemblies (hifiasm-meta, Hicanu or metaFlye). 97% threshold produced similar completeness evaluation results to those of 95%. 99% threshold resulted in more duplications, although also slightly improved the overall assembly completeness. Some contigs might be used more than once. We see little such duplication in the final result (i.e. checkM-passed MAGs). Note that the comparison is restricted within the collection of rescued circles candidates. A cycle that is very similar to a long circular contig would not be discarded because of so.

We run MetaBAT2 on raw contigs independent of circle rescue and then merge MetaBAT2 bins with rescued circles. We collect all rescued circles (set1) and remember the contigs used by them (setb). Next, MetaBAT2 bins that are at least 500kb, no more than 10Mb and do not only contain a single >1Mb circular contig were examined. If a bin has more than 1Mb of its contents coming from setb, or more than 10 of its contigs coming from setb, the bin is rejected. Otherwise we accept it into set2 and update setb with its contigs. We then collect linear contigs that are longer than 1Mb and not in setb into set3. All circular contigs longer than 1Mb form set4. The binning outcome (“merged MAGs” or “merged bins”) is the union of set1, 2, 3 and 4.

MAG quality evaluations

We first compared MAG quality brackets from rescued circles, MetaBAT2 bins and vamb bins (Table 1). There are 16 HiFi libraries available, all except one are gut materials (Table S1, S4). We follow the minimum information criteria about a single amplified genome (MISAG) convention [5] using checkM: “Near-complete” means $\geq 90\%$ completeness and $<5\%$ contamination. “High-quality” means $\geq 70\%$ completeness, $<10\%$ contamination but does not qualify for near-complete. “Medium-quality” means $\geq 50\%$ completeness, $\geq 50$ quality score but does not qualify for the above two. Quality score of a MAG is defined as “completeness$-5 \times$ contamination”. In terms of the yield in the near-complete quality bracket, the proposed topology-based rescue performed occasionally comparable to vamb, but was consistently worse than MetaBAT2 except for in env-digester-1. This was because binners were able to pull together long contigs that are not connected on the assembly graph to form a checkM-passing bin, while the rescue heuristic relies on graph traversal. Below we demonstrate the rescuing and the merge with regular MetaBAT2 bins using sheep-gut-1b, a diverse sheep fecal material sequenced with extraordinary depth and diversity [41]; see Table 2 for results of all samples available and Table S2 for binning details. We tried the heurstices on rust-mdbg [42] assembly graphs. The rescued circles can recover up to 1/3$\sim$1/4 of HiFi MAGs in the corresponding samples, although with more indels.
Table 1 CheckM evaluation of rescued circles, MetaBAT2 bins and vamb bins. Vamb requires at least 4096 contigs to run.

| sample          | binning method | near-complete bins | high-quality bins | near-complete orphaned >1Mb linear contig | high-quality orphaned >1Mb linear contig |
|-----------------|----------------|--------------------|------------------|------------------------------------------|------------------------------------------|
| sheep-gut-1a    | rescue         | 45                 | 35               | 18                                       | 17                                       |
|                 | metabat2       | 51                 | 34               | 22                                       | 15                                       |
|                 | vamb           | 31                 | 15               | 8                                        | 6                                        |
| sheep-gut-1b    | rescue         | 99                 | 15               | 85                                       | 59                                       |
|                 | metabat2       | 128                | 103              | 20                                       | 21                                       |
|                 | vamb           | 64                 | 71               | 79                                       | 62                                       |
| chicken-gut-1   | rescue         | 10                 | 18               | 8                                        | 7                                        |
|                 | metabat2       | 26                 | 18               | 0                                        | 3                                        |
|                 | vamb           | 18                 | 14               | 4                                        | 6                                        |
| env-digester-1  | rescue         | 12                 | 3                | 3                                        | 7                                        |
|                 | metabat2       | 9                  | 18               | 1                                        | 1                                        |
|                 | vamb           | 4                  | 14               | 4                                        | 7                                        |
| human-gut-1     | rescue         | 20                 | 3                | 13                                       | 15                                       |
|                 | metabat2       | 28                 | 32               | 8                                        | 10                                       |
|                 | vamb           | 18                 | 33               | 11                                       | 16                                       |
| human-gut-2     | rescue         | 21                 | 2                | 9                                        | 24                                       |
|                 | metabat2       | 33                 | 49               | 6                                        | 14                                       |
|                 | vamb           | 23                 | 41               | 8                                        | 21                                       |
| human-gut-3     | rescue         | 8                  | 1                | 4                                        | 3                                        |
|                 | metabat2       | 15                 | 4                | 0                                        | 1                                        |
|                 | vamb           | 8                  | 6                | 6                                        | 1                                        |
| human-gut-4     | rescue         | 7                  | 4                | 2                                        | 7                                        |
|                 | metabat2       | 24                 | 30               | 0                                        | 1                                        |
|                 | vamb           | 18                 | 28               | 2                                        | 6                                        |
| human-gut-5     | rescue         | 8                  | 3                | 1                                        | 3                                        |
|                 | metabat2       | 20                 | 13               | 1                                        | 0                                        |
|                 | vamb           | N/A                |                  |                                          |                                          |
| human-gut-6     | rescue         | 5                  | 3                | 2                                        | 2                                        |
|                 | metabat2       | 6                  | 10               | 1                                        | 2                                        |
|                 | vamb           | 8                  | 10               | 2                                        | 2                                        |
| human-gut-7     | rescue         | 5                  | 7                | 8                                        | 6                                        |
|                 | metabat2       | 22                 | 17               | 1                                        | 1                                        |
|                 | vamb           | 17                 | 14               | 5                                        | 6                                        |
| human-gut-8     | rescue         | 3                  | 3                | 1                                        | 4                                        |
|                 | metabat2       | 13                 | 12               | 0                                        | 0                                        |
|                 | vamb           | 8                  | 8                | 1                                        | 5                                        |
| human-gut-9     | rescue         | 4                  | 1                | 0                                        | 3                                        |
|                 | metabat2       | 10                 | 14               | 0                                        | 0                                        |
|                 | vamb           | 22                 | 11               | 0                                        | 0                                        |
| human-gut-10    | rescue         | 12                 | 3                | 4                                        | 6                                        |
|                 | metabat2       | 23                 | 21               | 0                                        | 1                                        |
|                 | vamb           | 19                 | 20               | 2                                        | 3                                        |
Table 2 Merging the rescued circles and MetaBAT2 bins. "90-5": near-complete. CheckM evaluation columns delimited by commas contain bin counts of in the order of near-complete, high-quality and medium-quality. Total MetaBAT2 bins did not double count the long circular contigs.

| sample            | #circular contig | #rescue | #metabat2 accept/all | checkM circular contig | checkM rescue | checkM metabat2 | checkM merged | #orphan | #present/miss 90-5 |
|-------------------|------------------|---------|----------------------|------------------------|---------------|-----------------|---------------|---------|-------------------|
| sheep-gut-1a      | 145              | 61      | 611/668              | 132,10,2               | 45,5,0        | 51,35,37        | 69,32,36      | 1/4     | 27/27             |
| sheep-gut-1b      | 279              | 161     | 1269/1431            | 249,22,2               | 99,15,2       | 130,104,111     | 188,102,96    | 4/25    | 37/41             |
| chicken-gut-1     | 73               | 12      | 332/379              | 62,10,1                | 10,0,0        | 26,18,14        | 28,18,14      | 0/0     | 8/8               |
| env-digester-1    | 21               | 41      | 292/316              | 18,2,0                 | 12,3,1        | 9,19,11         | 20,19,12      | 0/2     | 1/1               |
| human-gut-1       | 42               | 52      | 470/511              | 37,5,0                 | 20,3,2        | 28,32,29        | 37,28,29      | 2/22    | 8/11              |
| human-gut-2       | 46               | 39      | 545/561              | 37,7,1                 | 21,2,1        | 34,49,40        | 44,44,37      | 2/11    | 9/11              |
| human-gut-3       | 37               | 12      | 36/128               | 86,0,1                 | 8,1,0         | 15,4,8          | 17,4,8        | 0/4     | 5/6               |
| human-gut-4       | 18               | 37      | 234/267              | 16,2,0                 | 7,4,3         | 24,30,40        | 17,28,39      | 0/9     | 6/14              |
| human-gut-5       | 10               | 19      | 107/123              | 7,0,0                  | 8,3,0         | 20,13,8         | 18,15,7       | 0/4     | 8/10              |
| human-gut-6       | 7                | 15      | 76/93                | 4,1,2                  | 5,3,1         | 6,10,11         | 8,10,12       | 0/1     | 2/3               |
| human-gut-7       | 17               | 22      | 166/188              | 16,1,0                 | 5,7,1         | 22,17,18        | 21,18,17      | 0/2     | 5/6               |
| huamn-gut-8       | 4                | 15      | 112/124              | 3,0,0                  | 3,3,0         | 13,12,15        | 10,11,15      | 0/0     | 3/6               |
| human-gut-9       | 13               | 18      | 107/125              | 7,1,1                  | 4,1,3         | 10,14,19        | 7,9,21        | 0/1     | 4/7               |
| human-gut-10      | 23               | 30      | 180/206              | 19,3,0                 | 12,3,4        | 23,21,24        | 26,21,26      | 0/6     | 5/9               |
For sheep-gut-1b (Figure 2), the circle-rescue heuristic first considered 3340 cycles. After deduplication, 161 were reported, 99 of which were near-complete, 15 were high-quality and 1 was medium-quality. Additionally, 85 \( >1\)Mb linear contigs that were not used by medium-quality-and-above cycles were near complete. Only 25 of them could align more than 60\% of their length to a long circular contig or a high-quality-or-above rescued circle \((-c -xasm20\)\). The proportion of candidates failing the checkM quality check was comparable to traditional binning. For example, MetaBAT2 reported 438 bins that are at least 1Mb and less than 8Mb, 121 of them were near-complete, 80 were high-quality and 75 were medium-quality. We did not find obvious difference between the rescued circles that failed checkM, and the rescued circles in the better quality bracket, in terms of sequence homology to the long circular contigs. The true positive rate from rescued circles could be improved by discarding obviously wrong cycles (e.g. repeating similar subsequences), but it is not useful in practice, as MAGs will always be quality-controlled by checkM and other reference-based tools.

**Figure 2** Clustering of near-complete MAGs of sheep-gut-1b. The middle-left dense purple square represents Archaea. Mash distance more than 30\% are shown as 30\% for a better visual.
When merging the rescued circles with the regular MetaBAT2 binning (Table 2), most MetaBAT2 bins were accepted (1269/1431), which yielded 89 near-complete bins and 87 high-quality bins. 25 >1Mb linear contigs were accepted due to orphanage and all of them evaluated to be near-complete. Along with 279 >1Mb circular contigs (249 near-complete and 22 high-quality), this summed to 462 near-complete MAGs. 41 near-complete MetaBAT2 bins were rejected due to overlapping with rescued circles. We found 37 of them have counterparts in the collection of the rescued circles with less than 1% mash distance (30 had less than 0.1% mash distance match), effectively being replaced by them. Out of these 37 pairs, 11 rescued circles matches were better than the rejected MetaBAT2 bins in terms of better checkM completeness and/or contamination, 22 had identical checkM evaluations, 2 were slightly worse but comparable, and 2 were worse (rescued circle was wrong). Among the four MetaBAT2 bins without representation, 3 contained a single long linear contig (wrongly used by rescued circles), 1 contained two long linear contigs. Overall, we were able to recover more MAGs than MetaBAT2 alone, and replace many MetaBAT2 bins with pseudo-circles while having acceptable lose. We note that the lose would be larger in worse assemblies where the rescued circle is prone to make more errors, such as human-gut-4 shown in Table 2.

Since both circle-rescuing and the merging allow some contigs to be shared between bins, there could be duplications. To check this, we calculate pairwise mash distance between near-complete MAGs from the above. MetaBAT2 bin with more than 1 contig was concatenated with 31 N-base paddings for convenience. Mash sketch does hash k-mer with N-base instead of ignoring them, but this has little influence on the distance estimation here. There were 441 MAGs, therefore 97020 unique pairs. 2/97020 had less than 1% mash distance. One pair was between a linear long contig and a rescued circle’s path belonging to the same not fully resolved subgraph (0.7%; both 91% complete and 0.6% contaminated). The other pair was between two circular contigs (0.7%; both 100% complete and 1.1% contaminated). Overall, the MAG collection had little redundancy.

The MAG merging procedure described above does not look at existing gene annotations. It is possible to achieve better binning if we use checkM to guide binning (e.g. DAS Tools [36]). We showcase a simple checkM-guided bin merging to demonstrate the gap between merged MAGs and the almost-best-possible outcome in the near-complete quality bracket (Table 3). We say “almost” because we do not try to create new bins or swap bin contents, but just to avoid shadowing valid MetaBAT2 bins with wrong rescued circles. First, the circle-rescuing heuristics and MetaBAT2 binning were executed as described above. We then ran checkM on the rescued circles, MetaBAT2 bins and all 1Mb linear contigs separately. For each quality bracket, we first accept all rescued circles that qualify, then accept MetaBAT2 bins that qualify and have less than 100kb of its content used by rescued circles, followed by accepting >1Mb linear contigs that qualify and have not been used by the above two categories, and finally all >1Mb circular contigs that qualify.

**Sample representation completeness assessed using k-mer spectrum**
We borrow the k-mer spectrum plot presented such as in KAT [43] and mercury [44]. In single genome assemblies, the main interest is to grasp assembly redundancies, incompleteness, correction errors and phasing by looking at the plot. For metagenome
assembly, we focus on illustrating the first two aspects. Metagenome libraries have extremely high counts of low multiplicity k-mers, resulted from the combination of sequencing errors and reads from low prevalence species. We defined ratios based on k-mer counts in read occurrence ranges (rather than exact read occurrence values) to render the spectrum plot visually easier to interpret (Figure 3). In the modified k-mer spectrum plot, if haplotypes are not closely related, contain neglectable amount of repeats and the assembly is perfect, we expect to see the band representing 1× assembly multiplicity (saturated orange in such plots throughout this manuscript; “1× band” from now on) to dominate the plot within genome-wide read coverage range, and fall sharply outside of it. There will be very few k-mers with read multiplicities higher than genome-wide coverage due to shared sequences that are longer than the k-mers (see below). We also hope to see few k-mers that exist in the reads but not in contigs, i.e. 0× assembly multiplicity plotted saturated blue at the bottom of each plot, as they imply unassembled contents. If haplotypes are somewhat related, we expect to see several peaks formed by the 1× band due to some k-mers being shared by more than one haplotypes. As a real near-ideal empirical example, we compared the human HiFi datasets with HRGM [19] (Figure S3), which is a diverse yet non-redundant collection of 5414 species from human gut microbiome. This plot marks what the plot would look like for a reasonably good sample representation. If we can achieve a smaller 0× band than Figure S3 through de novo assembly, then the assembly offers no less information than comparing the sample to a comprehensive reference set.

Using the k-mer spectrum plots, we found that although hifiasm-meta could generate more near-complete circular contigs than previous studies, the circular contigs alone do not provide a complete view of the corresponding libraries (Figure 3, left column). Unresolved subgraphs in the primary assembly accounted for quite a few dominant haplotypes (Figure S4). All samples did not receive good representation from just the circular contigs except for sheep-gut-1a, but this could be greatly improved if the merged MAGs are used instead (Figure 3, right column), except for human-gut-9 and env-digester-1. In four libraries, merged MAG were close to the near-ideal situation demonstrated above: chicken-gut-1, human-gut-3, sheep-gut-1a and sheep-gut-1b. We extracted k-mers (n=31) of the 0× band and aligned them

| sample         | #near-complete | #high-quality | #medium-quality |
|----------------|----------------|---------------|-----------------|
| sheep-gut-1a   | 132+45+26+6=209 | 10+5+33+18=56 | 2+0+37+19=58 |
| sheep-gut-1b   | 249+99+94+19=461 | 22+15+101+30=168 | 2+2+110+49=163 |
| chicken-gut-1  | 62+10+18+0=90  | 10+0+18+7=35   | 1+0+14+13=28  |
| env-digester-1 | 18+12+8+0=38   | 2+3+19+5=29    | 0+1+11+5=17   |
| human-gut-1    | 37+20+21+7=85  | 5+3+31+15=54   | 0+2+29+30=61  |
| human-gut-2    | 37+21+25+5=88  | 7+1+48+19=76   | 1+1+39+34=75  |
| human-gut-3    | 36+8+10+0=54   | 0+1+4+2=7      | 1+0+8+6=15    |
| human-gut-4    | 16+7+18+0=41   | 2+4+30+4=40    | 0+3+39+10=52  |
| human-gut-5    | 7+8+15+0=30    | 0+3+13+3=19    | 0+0+8+17=25   |
| human-gut-6    | 4+5+4+1=14     | 1+3+9+3=16     | 2+1+11+2=16   |
| human-gut-7    | 16+5+19+1=41   | 1+7+14+6=28    | 0+1+18+3=22   |
| human-gut-8    | 3+3+11+0=17    | 0+3+11+3=17    | 0+0+5+5=20    |
| human-gut-9    | 7+4+6+0=17     | 1+1+14+1=17    | 1+3+19+4=27   |
| human-gut-10   | 19+12+18+0=49  | 3+3+20+6=32    | 0+4+23+7=34   |
Figure 3 K-mer spectrum plots of all samples. In each subplot: let $N_{x}^{(c)}$ be the number of k-mers occurring $\geq x$ times in reads and exactly $c$ times in contigs. Then $N_{x} = \sum_{c} N_{x}^{(c)}$ is the number of k-mers occurring $\geq x$ times in reads. The height of the blue area intersecting at $x$ equals $N_{x}^{(0)}/N_{x}$; the height of the orange area intersecting at $x$ equals $N_{x}^{(1)}/N_{x}$. Similarly we have green for $c = 2$, red for $c = 3$, etc. Intuitively speaking, a large orange region suggests a good assembly with most reads k-mers assembled into a single contig; a large blue region suggests an incomplete assembly that misses many high-abundance k-mers in reads; a large region of other colors suggests a potentially duplicated assembly with k-mers assembled into multiple contigs. We truncate at $x$ if there are less than 1 million k-mers occurring $x$ or more times in reads.
to MAGs in question with bwa aln [45] to see if they can be found by allowing a few mismatches or indels. This had very insignificant impact to the plot, however. We also examined the $2 \times$ or higher bands in the high read-multiplicity range by dumping all kmers from human-gut-10 that were from $2 \times$ to $15 \times$ bands, and had read multiplicities higher than 800. They were likely from ubiquitous genes (e.g. tRNAs) or horizontally transferable sequences (see Methods).

Cross validation with full length 16S rRNA compositional estimation
Complement to the the k-mer spectrum-based completeness evaluation is the 16S rRNA-based methods. Most HiFi reads are a few folds longer than the full length rRNA genes. This provides composition estimation for free. Predicting the rRNA genes is well-studied [46, 47]. The performance was reasonable in HiFi reads (e.g. for barrnap, around 13% 16S genes were marked as partial). 16S-based taxonomy annotation has been similarly extensively explored [48, 46, 49], but it suffers from reference scope bias. For example, the human-gut-9 library has 1.8M reads. 24445 16S sequences were identified (1.4%), 21954 of them (90.0%) were assigned with confident genus level annotation. In contrast, the env-digester-1 library has 1.0M reads. Similarly, 1.3% of them contain 16S genes, but only 21.2% which would have confident genus level annotation. This was not limited to 16S genes. The SRA minhash-based read taxonomy analysis only identified 41.78% reads as of cellular organism origin. Non-human gut samples were in the middleground: sheep-gut-1b has 1.2% reads containing 16S genes, with 47.3% of them confidently annotated to genus level.

Based on these observations, although reference-based methods are widely used in practice and could resolve to species level [12, 16], we use greedy incremental clustering to define OTUs with boundary set to mismatch sequence identity 99% [12] (see Methods). No assembly could recover all abundant OTUs, but those evaluated to be better in k-mer spectrum approach missed less (Figure 4; Figure S5 provides plots of all samples and other OTU boundaries). Some MAGs had more than one OTU assignment due to possible erroneous OTU clustering, 16S diversity within genome, or collapsing of very similar haplotypes during assembly. Missing high-prevalence OTUs are usually partially assembled, i.e. linear contigs or subgraphs that failed to form near-complete MAGs (see Methods).

Discussion
Drawbacks of short read metagenome assemblies and the justification of the cost of using accurate long reads.
With binning, the near-complete MAG yield from HiFi assemblies, usually 1~2 such MAGs per Gb per sample, is similar to or less than the yields of past short read studies (Figure S1) and currently at a substantially higher cost. It might seem that the only advantage of HiFi assembly is the contig continuity, which is important but not so much for gene-focused approaches. However, SR assemblies often miss the major species at MAG level, and what have been missed is not easy to guess. While this could be overcome if using a comprehensive catalog as reference and approach the sequencing library from alignment-based or pangenome point of view, actual studies tend to analyze or compare MAGs without accounting for possible missing
species. To illustrate the issue, we use Almeida et al.’s collection of human gut MAGs [18]. The authors assembled 13133 human gut metagenomic datasets from 75 studies with a unified pipeline (metaSPAdes and MetaBAT2, quality controlled by checkM), ruling out impact of any pipeline difference. We collected k-mer spectrum plots for 3490 assemblies (Table S3) that had at least 10 MAGs. We define a good assembly to have more than half of its 0x band below the 50% watermark, a nice assembly if using the 30% watermark, and a failed assembly otherwise. Only 0.46% assemblies were nice. 2.1% were good. The completeness of the final catalog might be at the mercy of assemblies of unrelated samples luckily recovering each others’ most abundant species. In contrast, except for env-digester-1 and human-gut-9, all other HiFi assemblies presented by the merged MAGs were nice. Moreover, combined with the representation-completeness evaluations from two different aspects, we hope that it will be possible to transform the ideal sequencing depth from simply common practices (e.g. ≤ 10 Gb for human gut samples) to be educated guesses. This would be especially important when faced with novel environmental samples.

Other than incomplete sample representation, due to short contig length and binning errors, SR MAGs are known to be less complete than they appear to be. Meziti et al. noticed that on average, 23% population core genes and 50% variable genes were missing from near 95% complete MAGs [50]. We found most SR MAGs from short read studies to be smaller than their counterparts found in the HiFi MAGs (Figure 5); mash distance <5%). 12 pairs between Almeida et al. MAGs and HiFi contigs had checkM completeness >99% and contamination <1%, yet the SR MAGs were smaller than the HiFi contigs for more than 100Kb. When compared to HRGM, this trend held. However, some HiFi circular contigs appeared smaller. HRGM is a non-redundant species-level collection. It is not clear whether we actually had smaller genomes (or if HRGM favored larger genomes when deduplicating), or the smaller size was caused by assembly errors. Anyway, this is outside the scope of this paper and we will investigate in future studies.

**Figure 4** OTU recovery of merged bins, showing sheep-gut-1b and env-digester-1, which evaluated poorly and good in k-mer spectrum plot, respectively.
Figure 5 Most near-complete circular contigs and rescued cycles of human gut samples are longer than assemblies in human gut short read MAG collection (Almeida et al.) or reference catalog (HRGM, the UHGG extended). We pair our MAGs with theirs if the pair’s mash distance is less than 5%. Reference MAGs are allowed to be used more than one time. If there is a tie between reference choices, we arbitrarily pick one. We have no requirements for MAG length when pairing. The Jaccard index (which is the basis of mash distance) already penalizes set size difference, i.e. comparing a set with its strict superset gives imperfect score.
Haplotypes represented by the rescued circles do not appear to be particularly harder to recover than those assembled into circular contigs in the general sample pool (Figure 2). At species level (mash distance <5%), most of our near-complete circular contigs (180/182; 163/180 if comparing to Almeida et al.) or near-complete rescued circles (93/93; 90/93 if Almeida et al.) from human samples had matches in HRGM. At strain level (mash distance <1%), 51/182 (28%) near-complete circular contigs and 16/93 (17%) near-complete rescued circles were found in Almeida et al.’s (difference not significant between the two: fisher’s exact, p-value=0.07).

Undocumented or unseen MAGs.
For our near-complete MAGs in human samples (8 individuals plus 2 4-pooled libraries), we noticed some species or genera were absent from public repositories or MAG collections from large scale studies, using a cutoff at mash dist 0.05 (roughly ≥ 95% ANI). 47 (11%) unseen if compared to only near-complete MAGs from Almeida et al, 17 (4%) if compared to all reported MAGs from them. 217 (51%) were not in refseq. For ≥ 1Mb circular contigs, that would be 33 (15%), 11 (5%) and 122 (56%), respectively. These ratios are consistent with previous study. Jin et al. reported [51] 475 high quality (checkM completeness ≥ 80%, contamination ≤ 5%, quality score ≥ 60) from pacbio-illumina hybrid assembly and binning. 4 MAGs were circular. The MAGs were compared to the UHGG dataset [52] with ANI cutoff at 95% and reported 24 MAGs as novel. In our comparison settings, the number of unseen MAGs are 43 (9%), 21 (4%) and 193 (41%), respectively. It is hard to verify whether these observations are due to true compositional diversity between individual samples [22] or representation-incomplete assembly of past projects. We did not find significant coverage difference between our MAGs that were unseen in previous works and the rest.

MAG catalogs other than the human gut microbiome’s are far from complete. We compared the chicken-gut-1 and the sheep samples to their relevant MAG catalogs, ICRGGC (chicken-gut-1) and PRJNA657473(ruminants), respectively. ICRGGC [53] contained 12339 MAGs derived from 799 samples. PRJNA657473 [54] contained 10371 MAGs derived from 370 samples (7 sample species, 10 gastrointestinal tract regions). Near-complete circular contigs and rescued circles from chicken-gut-1 found 55/62 (contig) and 8/10 (rescued) species-level matches, 7/62 (contig) and 0/10 (rescued) strain-level matches. sheep-gut-1b found no matches for 249 contigs and 99 rescued circles in both category, probably due to insufficient relevant reference.

Conclusions
In this study, we implemented a topology-based binning heuristic on the contig graph for hifiasm-meta and described two approaches, namely k-mer spectrum and species-evel OTUs based on full length 16S rRNAs, to evaluate an metagenome assembly’s completeness in terms of sample representation. We further showed that de novo HiFi assemblies plus binning would have the potential to be both genome-complete and representation-complete, bringing MAGs closer to their original goal which is to delegate their biosample with minimum bias. Note that inferring whether a sequencing library has sampled the microbiome of interest exhaustively is hard
and out of the scope of this work. There are other drawbacks, too. For example, the binning heuristic and the traditional binner still assume some reference bias due to being dependent on checkM as the final genome quality validation.

Nonetheless, we anticipate that high quality metagenome assemblies and further method improvements could transform previously inaccessible approaches, such as analyzing horizontal gene transfers, de novo variant calling in unusual samples and direct comparison between microbial communities.

Methods
Assembly, evaluation and simulation
We generated HiFi assemblies using hifiasm-meta r63 with default settings. See Table S5 for versions of tools used. Short read assemblies were downloaded from their releases. We used MetaBAT2 [32] with contig coverage. Contig coverage for MetaBAT2 and vamb binning was estimated with minimap2 alignment and MetaBAT2’s jgi module. Coverage: we ran minimap2 [55] with “minimap2 --sk19 -w10 -10G -g5k -r2k --lj-min-ratio 0.5 --A2 -B5 -05,56 -E4,1 -z400,50 --sam-hit-only contigs.fa reads.fa”. BAM file handling used SAMtools [56].

Coverage was estimated by “jgi_summarize_bam_contig_depths --outputDepth input.bam”. Binning: we ran MetaBAT2 using “metabat2 --seed 1 -i contigs.fa -a depth.txt”, and vamb using “vamb --p 48 --outdir ./ --fasta contigs.fa --jgi jgi_depth --minfasta 500000”. MetaBAT2’s random seed has little influence. We separate circular contigs of 1Mb or longer into a separate MAG if it is binned together with other contigs.

We used checkM module “lineage_wf” to evaluate MAG quality. Its outputs were formatted by “checkm qa -o 2” before parsing. We did not try DAS tools’s evaluation in this work, but it should give consistent but more generous results.

We did HiFi read simulation using PBSIM2 [57]: “pbsim2 --depth INT” “--sample-fastq sample.fp”. We generated “sample.fq”, the empirical error profile, by randomly sampling 100k reads from sheep-gut-1a. We used seqtk [https://github.com/lh3/seqtk]: “seqtk sample sheep-gut-1a.fq 100000 > out.fq”.

We forked yak [https://github.com/lh3/yak] for k-mer spectrum plot. The fork used k=31 rather than the k=27 default of KAT. This did not have significant impact on the plots; k=21 generated overall similar outputs as well.

We ran rust-mdBG with “-k 21 -l 14 --density 0.003 -p asm”, then “magic_simplify_meta asm” to generate the final assembly graph and the sequences per developer’s recommendation. A freestanding implementation of the circle-finding heuristics was used, and we used mash distance (cutoff: 90%) to compare between the reported circular paths to merged MAGs of hifiasm-meta’s. In sludge, humanO3 and sheepB, this reported 22, 22 and 120 rescued circles, respectively. We did not try to do metaBAT2 and bin merging because checkM was sensitive to indels.

Examining high read- and assembly-multiplicity kmers
We use human-gut-10 as an example. We identified kmers with at least 2x and up to 15x assembly multiplicity, and at least 800x read multiplicity, i.e. the right-most part of k-mer spectrum plot above 1x band. Their location on the contigs were
collected. We merged overlapping intervals and dumped the sequences. There were 5469 unique sequences (max length 18.6kb, N50 2.0kb). We randomly select 20 from these (“seqtk subseq in.fa 20”) and did BLAST (blastn web cgi, defaults) against nr/nt. All queries had full length BLAST hits with low sequence divergence and frequently overlap with genes encoding DNA-related enzymes, transposase and tRNA or rRNA (Table S6).

**16S rRNA methods**

We identified and annotated 16S rRNA genes from HiFi libraries with the following steps. First, HiFi reads that could aligned to SILVA reference were extracted, with base qualities stripped: “seqtk subseq hifi.fq <(minimap2 SILVA.fa hifi.fq | cut -f1 | uniq) | seqtk seq -A > SSUreads.fa”. We ran barrnap to identify rRNA genes: “barrnap --kingdom bac --outseq rRNA.fa SSUreads.fa”. INFERNAL cmsearch might identify a few more rRNAs than barrnap. We believe this would not have major influence on the conclusion based on previous observations. We then annotated rRNA genes with RDP classifier: “java -Xmx16g -jar RDPTools/classifier.jar classify -o RDP.tsv -h RDP.hier rRNA.fa”. We accept annotations of 16S rRNAs with genus scores of at least 0.9.

To define OTUs from HiFi reads, we first selected 16S genes not marked with “partial” from the barrnap. We used greedy incremental clustering: we initialize an empty collection $S$ to collect seed sequences. For each 16S gene $q$, if it could align to any sequence $s$ in $S$ with alignment block longer than 1000bp and at least 99% mismatch identity, it is assigned the same OTU label as $s$. (If multiple seeds are available, the one with highest identity will be chosen. If a tie, the seed is arbitrarily chosen from the bests.) Otherwise, $q$ is added to $S$ and assigned a new OTU label. Alignment is done with minimap2’s python binding, mappy, with “preset=map-hifi”. Alignment block length is given by “mappy.Alignment.blen”. Mismatch identity is calculated as “mappy.Alignment.mlen/mappy.Alignment.blen”. Assigning OTU label for an unseen 16S copy is done similarly. If a sequence can not align to any seed sequences, its OTU label is undefined.

There are two ways to assign OTU labels to MAGs: 1) collected reads belonging to contigs of a MAG and their OTU labels, or 2) identify 16S copies from contigs then assign labels. We did both and found them to be mostly consistent. We only considered near-complete MAGs.

When evaluating MAGs as in Figure 4 and Figure S5, we drop OTUs with less than 10 16S copies to rule out artifacts from sequencing errors and to ignore species with very low coverage. This was done after the OTU label assignment. A MAG could have more than one OTU assignment. It was difficult to distinguish wrong cases (i.e. suboptimal clustering result) from true cases, i.e. a genome having multiple distinct 16S copies, therefore we simply accept all OTU labels of a MAG. For example, if the read set yields 3 OTUs ($a,b$ and $c$), the assembly has a single MAG from which we identify three 16S copies that are labeled $a$, $a$, $b$. Then in the plot, both $a$ and $b$ would be colored as “seen in MAG”.

**Supplementary Information**

**Declarations**

**Ethics approval and consent to participate**

Not applicable.
Consent for publication
Not applicable.

Availability of data and materials
Hifiasm-meta is open source at https://github.com/xfengnefx/hifiasm-meta/. The yak fork used is at https://github.com/xfengnefx/yam. Scripts and assemblies are at https://github.com/xfengnefx/smt_mtgcomp and ftput://ftp.dfci.harvard.edu/pub/hli/hifiasm-meta/hifiasm-binning/asm/, respectively. Hifi libraries [29, 41, 58, 59] are all publicly available and can be found under their accession IDs (table S4). For convenience, in Table S3 we supply urls to the short read libraries we used when evaluating short read MAGs from Almeida et al (the accession IDs were originally available in their supplementary tables).

Competing interests
The authors declare that they have no competing interests.

Funding
This work was funded by National Human Genome Research Institute (NHGRI) R01HG010040 and U01HG010961, the Chan Zuckerberg Initiative and the Sloan foundation.

Authors’ contributions
HL and XF conceived the project, carried out analysis and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements
Not applicable.

Author details
1 Department of Data Sciences, Dana Farber Cancer Institute, Boston, US. 2 Department of Biomedical Informatics, Harvard Medical School, Boston, US.

References
1. Tully, B.J., Graham, E.D., Heidelberg, J.F.: The reconstruction of 2,631 draft metagenome-assembled genomes from the global oceans. Scientific data 5(1), 1–8 (2018)
2. Howe, A.C., Jansson, J.K., Malfatti, S.A., Tringe, S.G., Tiedje, J.M., Brown, C.T.: Tackling soil diversity with the assembly of large, complex metagenomes. Proceedings of the National Academy of Sciences 111(13), 4904–4909 (2014)
3. Kroeger, M.E., Delmont, T.O., Eren, A.M., Meyer, K.M., Guo, J., Khan, K., Rodrigues, J.L., Bohannan, B.J., Tringe, S.G., Borges, C.D., et al.: New biological insights into how deforestation in amazonia affects soil microbial communities using metagenomics and metagenome-assembled genomes. Frontiers in microbiology 9, 1635 (2018)
4. Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W.: CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25(7), 1043–1055 (2015)
5. Bowers, R.M., Kyrpides, N.C., Stepanauskas, R., Harmon-Smith, M., Doud, D., Reddy, T., Schulz, F., Jarett, J., Rivers, A.R., Eloe-Fadrosh, E.A., et al.: Minimum information about a single amplified genome (misag) and a metagenome-assembled genome (mimag) of bacteria and archaea. Nature biotechnology 35(8), 725–731 (2017)
6. Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K.L., Tyson, G.W., Nielsen, P.H.: Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nature biotechnology 31(6), 533–538 (2013)
7. Vicedomini, R., Quince, C., Darling, A.E., Chikhi, R.: Strainberry: automated strain separation in low-complexity metagenomes using long reads. Nature Communications 12(1), 1–14 (2021)
8. Nayfach, S., Shi, Z.J., Seshadri, R., Pollard, K.S., Kyrpides, N.C.: New insights from uncultivated genomes of the global human gut microbiome. Nature 568(7753), 505–510 (2019)
9. Luo, C., Tsanetzi, D., Kyrpides, N.C., Konstantinidis, K.T.: Individual genome assembly from complex community short-read metagenomic datasets. The ISME journal 6(4), 898–901 (2012)
10. Stackebrandt, E., Goebel, B.M.: Taxonomic note: a place for dna-dna reassociation and 16s rRNA sequence analysis in the present species definition in bacteriology. International journal of systematic and evolutionary microbiology 44(4), 846–849 (1994)
11. Brumfield, K.D., Hug, A., Colwell, R.R., Olds, J.L., Leddy, M.B.: Microbial resolution of whole genome shotgun and 16s rRNA metagenomic sequencing using publicly available neon data. PLoS One 15(2), 0228999 (2020)
12. Edgar, R.C.: Updating the 97% identity threshold for 16s rRNA gene clustering. Bioinformatics 34(14), 2371–2375 (2018)
13. Yuan, C., Lei, J., Cole, J., Sun, Y.: Reconstructing 16s rRNA genes in metagenomic data. Bioinformatics 31(12), 35–43 (2015)
14. Poretsky, R., Rodriguez-R, L.M., Luo, C., Tsanetzi, D., Konstantinidis, K.T.: Strengths and limitations of 16s rRNA gene amplification sequencing in revealing temporal microbial community dynamics. PloS one 9(4), 93827 (2014)
15. Kai, S., Matsuo, Y., Nakagawa, S., Kryukov, K., Matsukawa, S., Tanaka, H., Iwai, T., Imanishi, T., HirotA, K.: Rapid bacterial identification by direct pcr amplification of 16s rRNA genes using the minion™ nanopore sequencer. FEBS open bio 9(3), 548–557 (2019)

16. Johnson, J.S., Spakowitz, D.J., Hong, B.-Y., Petersen, L.M., Demkowicz, P., Chen, L., Leopold, S.R., Hanson, B.M., Agresta, H.O., Gerstein, M., et al.: Evaluation of 16s rRNA gene sequencing for species and strain-level microbiome analysis. Nature communications 10(1), 1–11 (2019)

17. Wang, W., Hu, H., Zijlstra, R.T., Zheng, J., Gänzle, M.G.: Metagenomic reconstructions of gut microbial metabolism in weanling pigs. Microbiome 7(1), 1–11 (2019)

18. Almeida, A., Mitchell, A.L., Boland, M., Forster, S.C., Gloor, G.B., Tarkowska, A., Lawley, T.D., Finn, R.D.: A new genomic blueprint of the human gut microbiota. Nature 568(7753), 499–504 (2019)

19. Kim, C.Y., Lee, M., Yang, S., Kim, K., Yong, D., Kim, H.R., Lee, I.: Human reference gut microbiome catalog including newly assembled genomes from under-represented asian metagenomes. Genome Medicine 13(1), 1–20 (2021)

20. Pasolli, E., Asnicar, F., Manara, S., Zolfi, M., Karcher, N., Armanini, F., Beghini, F., Manghi, P., Tett, A., Ghensi, P., et al.: Extensive unexplored human microbiome diversity revealed by over 150,000 genomes from metagenomes spanning age, geography, and lifestyle. Cell 176(3), 649–662 (2019)

21. Chen, C., Zhou, Y., Fu, H., Xiong, X., Fang, S., Jiang, H., Wu, J., Yang, H., Gao, J., Huang, L.: Expanded catalog of microbial genes and metagenome-assembled genomes from the pig gut microbiome. Nature communications 12(1), 1–13 (2021)

22. Visconti, A., Le Roy, C.I., Rosa, F., Rossi, N., Martin, T.C., Mohney, R.P., Li, W., de Rinaldis, E., Bell, J.T., Venter, J.C., et al.: Interplay between the human gut microbiome and host metabolism. Nature communications 10(1), 1–10 (2019)

23. Liu, P., Hu, S., He, Z., Feng, C., Dong, G., An, S., Liu, R., Xu, F., Chen, Y., Ying, X.: Towards strain-level complexity: Sequencing depth required for comprehensive single-nucleotide polymorphism analysis of the human gut microbiome. Frontiers in Microbiology 13 (2022)

24. Hillmann, B., Al-Ghalith, G.A., Shields-Cutler, R.R., Zhu, Q., Gohl, D.M., Beckman, K.B., Knight, R., Knights, D.: Evaluating the information content of shallow shotgun metagenomics. Msystems 3(6), 00069-18 (2018)

25. Institute, D.I.G. https: //www.ncbi.nlm.nih.gov/proj ect?term = wetland & microbial + communities + from + Twistell + Island (2011)

26. Kanehisa, M., Sato, Y., Morishima, K.: Blastkoala and ghostkoala: Kegg tools for functional characterization of genome and metagenome sequences. Journal of molecular biology 428(4), 726–731 (2016)

27. Frioux, C., Singh, D., Korschmaros, T., Hildebrand, F.: From bag-of-genes to bag-of-genomes: metabolic modelling of communities in the era of metagenome-assembled genomes. Computational and Structural Biotechnology Journal 18, 1722–1734 (2020)

28. Laudadio, I., Fults, V., Palone, F., Stornati, L., Cucchiara, S., Carissimi, C.: Quantitative assessment of shotgun metagenomics and 16s rDNA amplicon sequencing in the study of human gut microbiome. OMiCS: A Journal of Integrative Biology 22(4), 248–254 (2018)

29. Kolmogorov, M., Bickhart, D.M., Behsaz, B., Gurevich, A., Rayko, M., Shin, S.B., Kuhn, K., Yuan, J., Polevиков, E., Smith, T.P.L., Pevzner, P.A.: metaFlye: scalable long-read metagenome assembly using repeat graphs. Nat. Methods 17(11), 1103–1110 (2020)

30. Nork, S., Walenz, B.P., Rhee, A., Vollger, M.R., Logsdon, G.A., Grothe, R., Miga, K.H., Eichler, E.E., Phillippy, A.M., Koren, S.: HiCanu: accurate assembly of segmental duplications, satellites, and allelic variants from high-fidelity long reads. Genome Res. 30(9), 1291–1305 (2020)

31. Feng, X., Cheng, H., Portik, D., Li, H.: Metagenome assembly of high-fidelity long reads with hifiasm-meta. Bioinformatics 1–18 (2014)

32. Kang, D.D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., Wang, Z.: MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. PeerJ 7, 7359 (2019)

33. Nissen, J.N., Johansen, J., Allesøe, R.L., Sønderby, C.K., Armenteros, J.J.A., Grünbech, C.H., Jensen, L.J., Nielsen, H.B., Petersen, T.N., Winther, O., et al.: Improved metagenome binning and assembly using deep variational autoencoders. Nature biotechnology 39(5), 555–560 (2021)

34. Mallawaarachchi, V., Wickramarachchi, A., Lin, Y.: Graphbin: refined binning of metagenomic contigs using assembly graphs. Bioinformatics 36(11), 3307–3313 (2020)

35. Wu, Y.-W., Tang, Y.-H., Tringe, S.G., Simmons, B.A., Singer, S.W.: Maxbin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. Microbiome 2(1), 1–18 (2014)

36. Sieber, C.M., Probst, A.J., Sharrar, A., Thomas, B.C., Hess, M., Tringe, S.G., Banfield, J.F.: Recovery of genomes from metagenomes via a dereplication, aggregation and scoring strategy. Nature microbiology 3(7), 836–843 (2018)

37. Wick, R.R., Schultz, M.B., Zobel, J., Holt, K.E.: Bandage: interactive visualization of de novo genome assemblies. Bioinformatics 31(20), 3350–3352 (2015)

38. Ondov, B.D., Treangen, T.J., Melsted, P., Mallon, A.B., Bergman, N.H., Koren, S., Phillippy, A.M.: Mash: fast genome and metagenome distance estimation using minhash. Genome biology 17(1), 1–14 (2016)

39. Jain, C., Rodriguez-R, L.M., Phillippy, A.M., Konstantinidis, K.T., Aluru, S.: High throughput ani analysis of complex microbial communities. Nature communications 9(1), 1–8 (2018)

40. Parks, D.H., Chuvochina, M., Chaumeil, P.-A., Rinke, C., Mussig, A.J., Hugenholtz, P.: A complete domain-to-species taxonomy for bacteria and archaea. Nature biotechnology 38(9), 1079–1086 (2020)

41. Bickhart, D.M., Kolmogorov, M., Tseng, E., Portik, D.M., Korobeynikov, A., Tolstoguzov, I., Ustynyuk, G., Liachko, I., Sullivan, S.T., Shin, S.B., et al.: Generating lineage-resolved, complete metagenome-assembled genomes from complex microbial communities. Nature biotechnology, 1–9 (2022)

42. Ekim, B., Berger, B., Chikhi, R.: Minimizer-space de bruijn graphs: Whole-genome assembly of long reads in minutes on a personal computer. Cell systems 12(10), 958–968 (2021)
Tables

Additional Files

Table S1. Supplementary tables and data release used a different sample naming convention. This file provides the name mapping.

Table S2. Binning information and evaluation of bins.

A tab-delimited table.

Supplementary Figure 1 Almeida et al. unified assembly and MAG quality evaluations for 13,133 human gut metagenomic datasets from 75 short-read-based studies. Most libraries are smaller than 10Gbp (99.2%), and per Gbp MAG recovery visually negatively correlated with library size. For an “average” library, we expect 1-2 near-complete MAGs. HiFi assemblies provide closed contigs and much lower intra-MAG heterozygosity (0.06% near-complete circular contigs have \(\geq 25\%\) checkM heterogenous score, versus 23.8% for SR) at similar per Gbp yields.
Table S3. The 3490 samples from Almeida et al. used in this manuscript. List of files (sra ftq).

Table S4. Sample and HiFi library information.
Table S5. List of tools their versions used in this manuscript.
Table S6. BLAST result summary of 20 high read- and contig- multiplicity k-mers intervals.

Supplementary Figure 2 A few runs from short read metagenome assembly studies achieved reasonable sample representation. However, they were fragmented and larger library size did not imply a better outcome.
Supplementary Figure 3 K-mer spectrum plots using HRGM assemblies as the MAGs, and HiFi reads as the library. Intend to show how good would a relatively complete library represent a new sample.
Supplementary Figure 4  Coverage of sheep-gut-1b merged MAGs by category.
Supplementary Figure 5 OTU recovery in samples, with OTU boundary set at 99%, 95% and 90%. The plots for human-gut-1 and human-gut-2 were omitted, because the each of these libraries are a pool of four different samples.