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The metagenome of the female upper reproductive tract

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Abstract:	Background The human uterus is traditionally believed to be sterile, while the vaginal microbiota plays important roles in fending off pathogens. Emerging evidence demonstrates the presence of bacteria beyond the vagina. However, a microbiome-wide metagenomic analysis identifying the overall microorganism communities has been lacking. Results We performed shotgun-sequencing by Illumina platform of 52 samples from the cervical canal and the peritoneal fluid of Chinese women in reproductive age. Direct annotation of sequencing reads identified the taxonomy of bacteria, archaea, fungi and viruses, confirming and extending the results from our previous study. We replicated the findings in another 24 samples from the vagina, the cervical canal, the uterus and the peritoneal fluid using BGISEQ-500 platform, revealing that microorganisms in the samples from the same individual were largely shared in the whole reproductive tract. Over 99% human sequences were detected in the 20GB raw data. After filtering, vaginal microorganisms were well covered in the generated reproductive tract gene catalogue, while the more diverse upper reproductive tract microbiota might need greater depth of sequencing and more samples to meet the full coverage scale. Conclusions Microbiota in unprecedented data for unchartered body site, female upper reproductive tract, were analyzed in this study. The community results indicated that an intra-individual continuum of all types of microorganisms gradually changed from the vagina to the peritoneal fluid. A framework was also established in this study aiming at understanding the implications of the composition and functional potential of this distinct microbial ecosystem in relation to health and disease.
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The metagenome of the female upper reproductive tract

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Abstract

Background

The human uterus is traditionally believed to be sterile, while the vaginal microbiota plays important roles in fending off pathogens. Emerging evidence demonstrates the presence of bacteria beyond the vagina. However, a microbiome-wide metagenomic analysis identifying the overall microorganism communities has been lacking.

Results

We performed shotgun-sequencing by Illumina platform of 52 samples from the cervical canal and the peritoneal fluid of Chinese women in reproductive age. Direct annotation of sequencing reads identified the taxonomy of bacteria, archaea, fungi and viruses, confirming and extending the results from our previous study. We replicated the findings in another 24 samples from the vagina, the cervical canal, the uterus and the peritoneal fluid using BGISEQ-500 platform, revealing that microorganisms in the samples from the same individual were largely shared in the whole reproductive tract. Over 99% human sequences were detected in the 20GB raw data. After filtering, vaginal microorganisms were well covered in the generated reproductive tract gene catalogue, while the more diverse upper reproductive tract microbiota might need greater depth of sequencing and more samples to meet the full coverage scale.

Conclusions

Microbiota in unprecedented data for unchartered body site, female upper reproductive tract, were analyzed in this study. The community results indicated that an intra-individual continuum of all types of microorganisms gradually changed from the vagina to the peritoneal

fluid. A framework was also established in this study aiming at understanding the implications of the composition and functional potential of this distinct microbial ecosystem in relation to health and disease.

Keywords

Metagenomics, Female upper reproductive tract

Background

As humans evolved, the female reproductive tract has formed complex and unique structures such as the uterus, cervix and the vagina. The human vagina hosts trillions of bacteria that can significantly impact the health of women and their neonates. The cervix has been regarded to be a perfect barrier between the vagina and uterus leading to the assumption that the upper reproductive tract functions in a sterile environment. However, judging from evidence in insects and other animals, humans are probably no exception with regard to vertical transmission of the mothers' microbiota before birth [1]. Thus, in humans, bacterial DNA has been detected in the placenta [2,3]. Based on our recent analyses using 16S rRNA amplicon sequencing, the upper reproductive tract, including cervix, uterus, fallopian tubes and peritoneal fluid harbor diverse communities of bacteria, though at low abundance [4].

Recently, the studies of female reproductive tract microbiota have mainly focused on the vagina using 16S rRNA amplicon sequencing [5–7]. Studies using 16S rRNA gene amplicon sequencing have limitations in relation to lower taxonomic resolution and the lack of ability to perform species-specific functional inference. Metagenomic shotgun sequencing can address these limitations, but only a few studies have applied metagenomic shotgun sequencing on the

vaginal microbiota [8], and no studies have characterized the compositional range of the upper reproductive tract microbiome using metagenomic analysis. The present study is the first to provide metagenomic data from the female upper reproductive tract.

Data description

Samples of six locations (CL, lower third of vagina; CU, posterior fornix; CV, cervical mucus drawn from the cervical canal; ET, endometrium; FLL and FRL, left and right fallopian tubes; PF, peritoneal fluid from the pouch of Douglas) throughout the female reproductive tract from 137 Chinese women of reproductive age, undergoing surgery for conditions not known to involve infection (**Supplementary Table 1**) were collected for this study. 16S rRNA gene amplicon sequencing was performed on 665 of these samples. The results from 476 of these have been published previously [4], and those from the remaining 189 were presented in this study. Two samples (1 CV and 1 CU) were subjected to shotgun sequencing with or without prior removal of human DNA using a commercial kit to test the experimental effect of host sequencing removing (refer to **Methods** section). Then, 25 PF and 25 CV samples were sequenced on the Illumina HiSeq platform using 100 bp paired-end (PE) sequencing (the stringent selection rules of samples, see **Methods** and **Supplementary Fig. 2** for details). For these 52 samples, 20GB of raw data per sample, corresponding to a total of 0.99 TB were generated. Additionally, intra-individual similarity in the vagino-uterine microbiota were also examined basing on 24 samples from different sites of the reproductive tract (CL, CU, CV, ET, PF) in 6 women. These samples were sequenced on the BGISEQ-500 sequencer using 100 bp single-end (SE) sequencing and generated 60GB of raw data per sample, totaling 1.40

TB. The dataset after filtering out low-quality and host reads (refer to **Methods** section) was available on EBI database with the accession number PRJEB24147.

Analyses and Discussion

Metagenomic sequencing

According to shotgun-sequencing of vaginal samples by the Human Microbiome Project (HMP) and of placental samples by Aagaard et al., over 90% of the sequences were derived from human host DNA [2,9]. To overcome this problem, we first tested a commercial kit that removes human DNA by binding and precipitating CpG-methylated DNA. Unfortunately, after the kit treatment, a considerable amount (99.9% for CV sample and 79% for CU sample) of host DNA still remained (**Supplementary Fig. 1a**). Besides, the bacteria compositions were varied by kit treatment when comparing with the control group (**Supplementary Fig. 1b**). We therefore abandoned the strategy of host DNA removal prior to shotgun metagenomics sequencing.

The sample selection was founded on the data from CV and PF samples [4], which we identified as robust representations of the overall samples. Since higher amounts of DNA is required for shotgun-sequencing results, a more stringent rule was set as the following two criteria: individual sub-clusters representation and sufficient DNA amount (see details in **Methods** section). To follow the former criterion, clustering results based on the relative abundances of OTUs in the PF and CV samples showed that the samples marked with red (all containing DNA > 1 μg) were well distributed amongst all collected samples (**Supplementary Fig. 2**), so these were selected for shotgun-sequencing in this study. As a

result, 25 PF and 25 CV samples were selected for sequencing using the Illumina HiSeq 4000 platform. After quality control, high-quality reads were aligned to hg 19 using SOAP and GRCh38 using DeconSeq to remove human reads (see details in **Methods** section). The average host contamination rate of 99.72% for CV and 99.93% for PF (**Supplementary Table 2**), which were lower than that previously reported for placenta samples [2].

The findings further expanded by inclusion of additional 24 samples subjected to sequencing on the BGISEQ-500 platform, in which we also examined the intra-individual similarity in the vagino-uterine microbiota based on samples from different sites of the reproductive tract (CL, CU, CV, ET, PF). The average host contamination rate for vagina (CL, CU) samples was 96.55%, and lower than those of the CV, ET and PF samples, which all above 99.5% (**Supplementary Table 2**).

A diverse microbiome in the cervical canal and the peritoneal fluid of reproductive age women

To obtain an overview of the overall composition of the vagino-uterine microbiome, we used Kraken to directly assign sequencing reads to all types of microbial taxa [10]. The dominant *Lactobacillus* spp. in CV and *Pseudomonas* spp. in PF were detected in the present study and in corresponded with the previous study. In addition, the microbiome that comprise methane-producing archaea, yeasts, herpesviruses, papillomaviruses, and bacteriophages were also founded (**Fig. 1a, b**).

The abundance of these taxonomic units varied among samples, and those constituting more than 0.1% of the total reads number were identified in the CV and PF samples from the same individual (**Fig. 1c**).

To gain further insight into compositional similarities of the microbiota at different sites of the reproductive tract in the same individual, we selected taxa at the family level which fulfilled two criteria: they were presented in at least two sites of the same individual and the relative abundance was higher than 0.1%. Taxa fulfilling these criteria made up more than 45% of the microorganisms presented in the samples across the 6 individuals subjected for this detailed analysis (**Fig. 2**). Lactobacillaceae or Bifidobacteriaceae dominated in vagina (CL and CU), but not in the upper reproductive tract, where microorganisms such as Pseudomonadaceae, Propionibacteriaceae, Streptococcaceae and Moraxellaceae constituted a notable fraction of the microbiota. In addition, eukaryotes, viruses and archaea, such as Saccharomycetaceae, Herpesviridae, Ferroplasmaceae were also found in the female reproductive tract. The results at the bacterial level are in keeping with our findings in a recent study [4], and the current data further demonstrates an intra-individual continuum of all types of microorganisms that gradually changes from the vagina to the peritoneal fluid.

Genes from the vagino-uterine microbiota

Reference gene catalogs have greatly facilitated analyses of the microbiome, especially the human gut microbiome [11–13]. Here, we established the first gene catalog of the microbiome of the female upper reproductive tract, which comprising 60,699 genes.

Rarefaction analysis based on gene number revealed a curve approaching saturation with about 23 CV samples (**Fig. 3**). By contrast, rarefaction analysis based on gene numbers in PF samples revealed a curve far from saturation, possibly due to a more diverse microbiota in PF. Therefore, with 20GB sequences per sample, vaginal bacteria could be well covered, whereas characterization of bacteria from the upper reproductive tract would require a higher amount

of sequences and more samples.

We annotated the genes in the gene catalog according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) [14]. The matched genes in PF (15,316 genes) were all covered within CV (39,087 genes). Comparing CV and PF in the distribution of KEGG pathways, PF showed a greater proportion of genes in carbohydrate metabolism, replication and repair, membrane transport and drug resistance, whereas the genes involved in translation, energy metabolism and metabolism of cofactors and vitamins were enriched in CV (**Supplementary Fig. 3**). In KO modules, CV showed enrichment of transport systems for thiamine, cystine, teichoic acid, taurine and putative ABC transport systems compared to PF. Regulatory systems of aerobic and anaerobic respiration, osmotic stress response and multicellular behavior control also enriched in CV (**Supplementary Table 3**).

Methods

Sample description

A total of 137 Chinese women of reproductive age, undergoing surgery for conditions not known to involve infection (hysteromyoma, adenomyosis, endometriosis, and salpingemphraxis) were enrolled in this study (**Supplementary Table 1**). Samples were taken from the CL, CU and CV on the day of the clinical visit without any prior disturbance. Depending on the clinical conditions, laparoscopy or laparotomy were performed, and samples from the ET, FLL, FRL and PF were taken during surgery (**Supplementary Table 1**). The study was approved by the institutional review boards at Peking University Shenzhen Hospital and BGI-Shenzhen, and all women provided informed written consent. The subject

exclusion criteria, sampling method and DNA extraction can be found in [4].

To test the effect of experimental removal of human DNA, one CU sample and one CV sample were used to shotgun sequencing on Illumina HiSeq2000 platform with or without prior removal of human DNA, respectively. The NEBNext Microbiome DNA Enrichment Kit was used here according to the manufacturer's instructions with a total of 10 μg input DNA per sample.

Then we made a prior selection of samples to undergo shotgun-sequencing. The selection was founded on the data from CV and PF samples [4] based on the following two criteria: i) samples should represent individual sub-clusters when subjected to clustering based on relative abundances of operational taxonomic units (OTUs) from 16S rRNA gene amplicon sequencing; ii) the amount of DNA should be above 1 μg. The samples with good scattering in different clusters based on the relative abundances of OTUs in the PF and CV samples were selected for shotgun-sequencing on Illumina HiSeq4000 platform.

We replicated the findings in another 24 samples on the BGISEQ-500 platform, where additional sites (CL, CU, CV, ET and PF) of 6 women were moreover involved. To meet the need of library construction, the amount of DNA in the all 24 samples were above 1 μg. And three qualified samples for each woman were set as a threshold.

Metagenomic shotgun sequencing

Library construction and shotgun sequencing using Illumina HiSeq2000/4000 platforms (insert size 350 bp; 100 bp of paired-end reads; two replicate libraries were constructed for each lane.) and BGISEQ-500 (100 bp of single-end reads; one library was constructed for

each lane) were performed as previously described [15]. The quality control of sequencing data from the HiSeq and BGISEQ platforms were also followed this study. Then, human sequences were eliminated by alignment to the hg19 reference genome using SOAP2.22. As the resulting data still contained human sequences, a more stringent procedure using DeconSeq by aligning data to the GRCh38 reference genome was applied [16].

Taxonomic assignment of sequencing reads

High-quality, non-human sequences were tentatively assigned to microbial taxa using Kraken with default parameters [10]. For pair-end reads Kraken concatenated the pairs together with a single N between the sequences automatically with default parameters and the manual clarified that this software raised the sensitivity by about 3 percentage points over classifying the sequences as single-end reads.

Construction of a gene catalog

The high-quality, non-human sequencing reads of 52 samples sequenced by Illumina HiSeq platforms were *de novo* assembled into contigs using IDBA-UD [17]. We used the same strategy as previous study [12,13], where genes were predicted from the contigs by MetaGeneMark [18], and highly similar genes (95% identity, 90% overlap) were removed as redundancy using CD-HIT [19]. Functional annotations were made by BLASTP (v2.2.24) based on KEGG (v76) databases [14].

Author's contributions

H.J. and R.W. conceived and directed the project. W.W., J.D., L.Z., H.D., H.T., and R.W. performed the clinical diagnosis, sample collection. C.C., Z.W., F.L., and L.H. performed the

bioinformatic analyses and prepared display items. C.C., F.L., Z.W., X.Z., J.L. and H.J. wrote the first version of the manuscript. L.M., S.B. and K.K. revised the manuscript. All authors contributed to the final revision of the manuscript.

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Competing financial interests

The authors declare no competing financial interests.

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Figure legends

Figure 1: The overall microbiome composition of the cervical canal and the peritoneal fluid of reproductive age women. Cumulative bar charts of the main taxa at domain (**a**) and family (**b**) levels in CV and PF samples. (**c**) Compositional overlap at family level of CV and PF samples from the same individuals. Relative number of reads was calculated as $N_p = \frac{a_p}{a_t} \times m$, where a_p is the number of reads within p taxa in a sample. a_t is the total number of reads within a sample, m is median number of reads within all 50 samples. When p taxa is shared by CV and PF samples from the same individuals, and at the same time both N_p values are higher than $0.1\% \times m$, the p taxa is included in the cumulative bar charts. Taxa names (**b**, **c**) in black, purple, and blue denote bacteria, eukaryote and viruses, respectively.

Figure 2: Composition of the vagino-uterine microbiota. (**a**, **c**, **e**, **g**, **i**, **k**) Venn diagram depicting shared taxa at the family levels in samples collected at different sites in the same individual. (**b**, **d**, **f**, **h**, **k**, **l**) Cumulative bar charts of the taxa with relative abundance higher than 0.1% and present in at least two sites of the same individual. Taxa names (**b**, **c**) in black, purple, blue, and grey denote bacteria, eukaryote, viruses and archaea, respectively.

Figure 3: Rarefaction of microbial gene content in CV (a) and PF (b) samples. The number of genes in each group was calculated after 100 random samplings with replacement. Boxes denote the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively) and the line inside denotes the median. Whiskers denote the lowest and highest values within 1.5 times IQR from the first and third quartiles, respectively.

313 Circles denote outliers beyond the whiskers.

314 Supplementary Figure legends

315 **Supplementary Figure 1: Evaluation of the NEBNext Microbiome DNA Enrichment Kit**
316 **by two comparative strategies.** Sample names suffixed by "-HR" represent DNA samples
317 that were treated with the kit for removal of host DNA before shotgun sequencing, while
318 sample names suffixed by A represent DNA samples that were subjected to shotgun
319 sequencing directly (**a**). The table data shows the obtained read number, and remaining reads
320 after removal of host DNA reads in the two samples. **b**) Influence of host DNA presence on
321 bacterial DNA identification during shotgun sequencing. The plots display the compositional
322 difference amongst major bacteria genera in samples with and without (-HR) host DNA
323 presence. Data were analyzed by mapping reads to the ICG bacterial reference gene catalog
324 [12].

325 **Supplementary Figure 2: Samples selected for metagenomic sequencing.** Average linkage
326 clustering of CV (**a**) and PF (**b**) samples based on the relative abundances of OTUs. Samples
327 which represent individual sub-clusters and hold DNA amounts above 1 μg were selected for
328 shotgun-sequencing (red).

329 **Supplementary Figure 3: KEGG pathway classification of the vagino-uterine**
330 **microbiome.** Comparison of CV (red) and PF (blue) data based on KEGG annotation, which
331 emphasizes functional similarity of the CV and PF microbiota.

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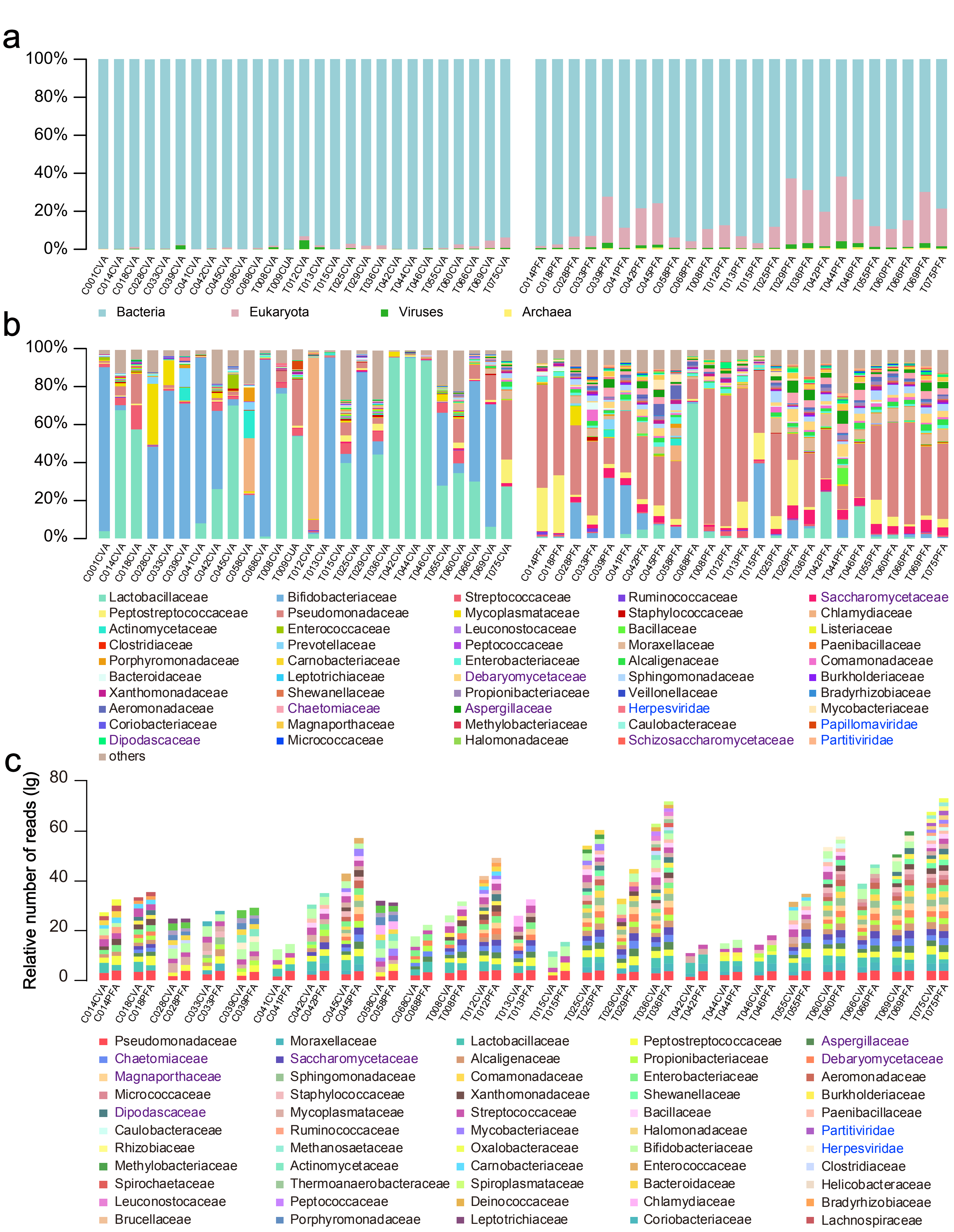

Figure 2

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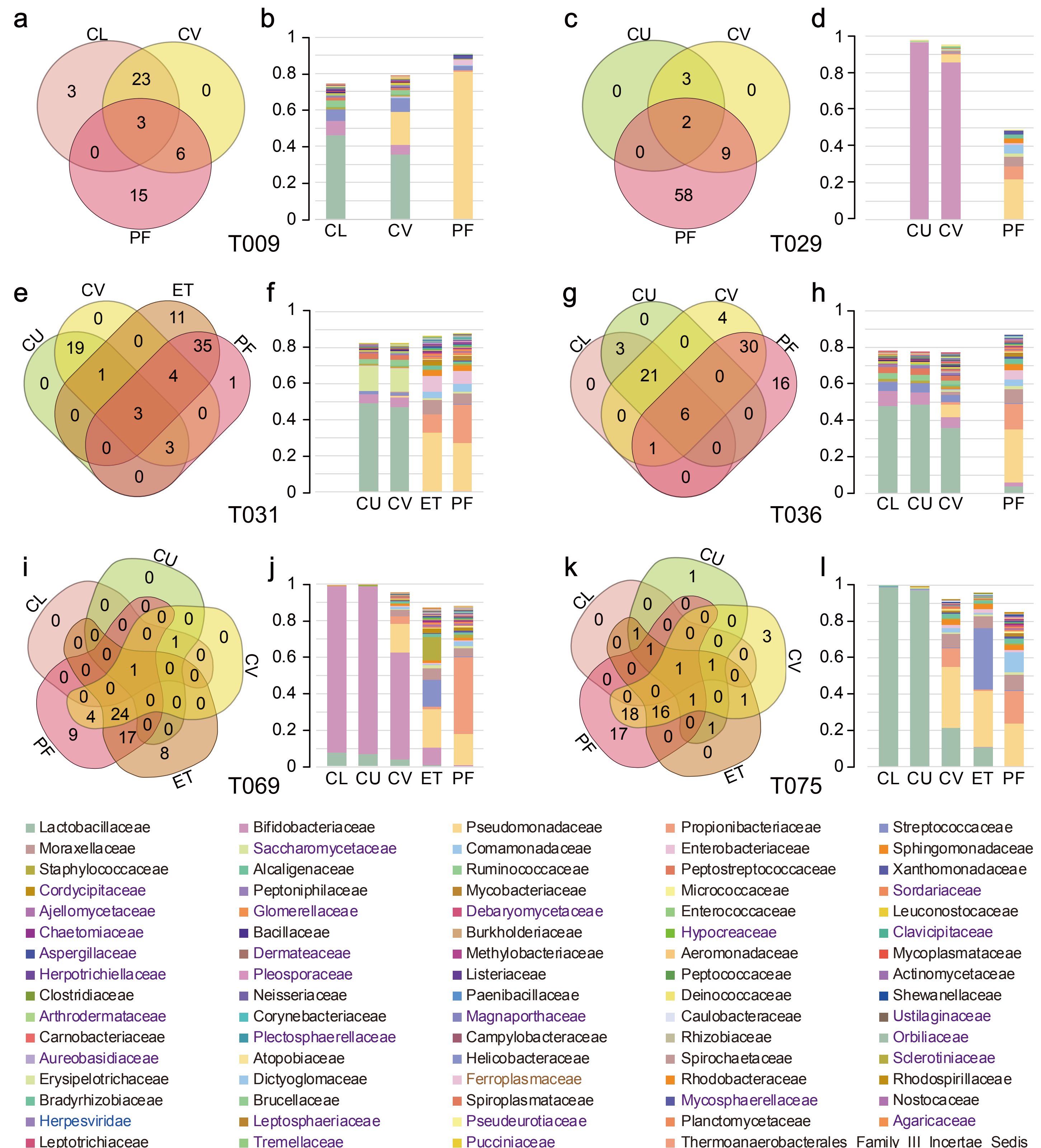

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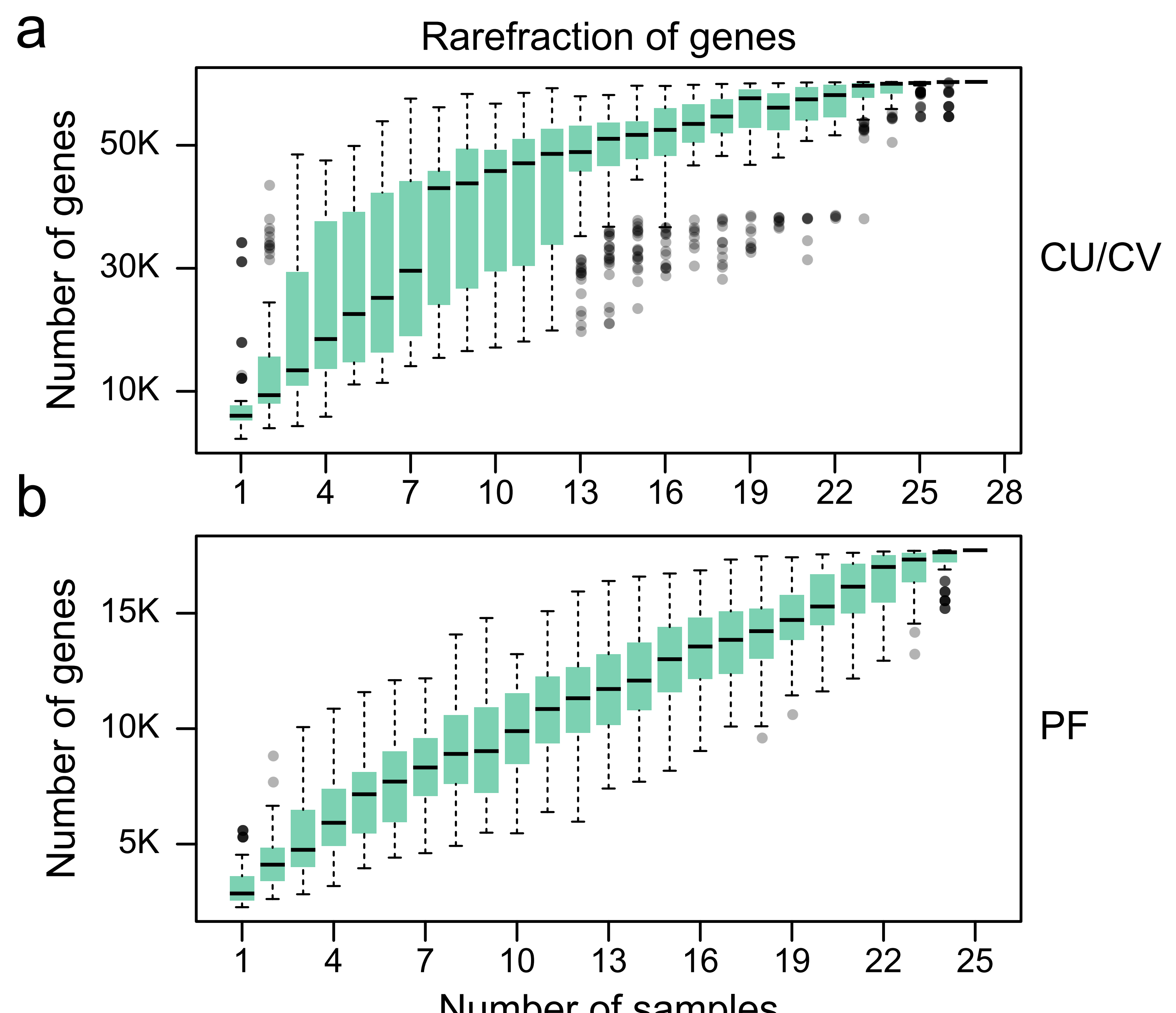
Figure 3



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