Microbial signatures in the lower airways of mechanically ventilated COVID19 patients associated with poor clinical outcome

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Supplementary Results:

Cohort description

Supplementary Tables 1 and **2** compare similar data across all 589 subjects, divided per site and sub-cohorts. Patients at the Manhattan campus who underwent bronchoscopy were younger, had lower body mass index (BMI), and a lower prevalence of chronic obstructive pulmonary disease (COPD; **Supplementary Table 1**). Among the cohort that provided lower airway samples through bronchoscopy, 37% of the subjects were successfully weaned within 28 days of initiation of MV and survived hospitalization, 39% required prolonged MV but survived hospitalization, and 24% died.

Mortality among those in the no-bronchoscopy cohort was 77%. In the overall NYU cohort, higher age and BMI were associated with increased mortality (**Supplementary Table 2**). There was a similar, albeit non-significant, trend for the bronchoscopy cohort. Among the clinical characteristics of this cohort, patients within the deceased group more commonly had a past medical history of chronic kidney disease and cerebrovascular accident.

Study patients were admitted during the first wave of the pandemic in the US, prior to current standardized management of COVID-19. Within the bronchoscopy cohort, more than 90% of the subjects received hydroxychloroquine and anticoagulation (therapeutic dose), 69% received corticosteroids, 41% received tocilizumab (anti-Interleukin (IL)-6 receptor monoclonal antibody), 21% required dialysis, and 18.9% were started on veno-venous extracorporeal membrane oxygenation (ECMO) (**Table 1**). Antimicrobial therapy included use of antivirals (lopinavir/ritonavir in 16% and remdesivir in 10%), antifungals (fluconazole in 40% and micafungin in 57%), and antibiotics (any, in 90% of the subjects).

During their hospitalization, most patients had respiratory and/or blood specimens collected for bacterial cultures (**Table 1** and **Supplementary Table 1**). The proportions

of positive bacterial respiratory cultures and blood cultures were 73% and 43%, respectively.

Microbial community structure of the lower & upper airways

Among the top 10 taxa across lower and upper airway samples were *Staphylococcus aureus*, *Salmonella enterica*, *Burkholderia dolosa*, and *Klebsiella variicola*. *Candida albicans* only ranked #77 in the BAL while it was ranked 5th in the metatranscriptome data indicating that while present at low relative abundance, it was highly active (**Supplementary Table 4**). *K. variicola*, while prevalent at a high relative abundance (#4 in BAL, and #5 in the upper airways) in patients of this cohort, its ranking in the RNAseq data was not among the top 50, indicating that it was not as active functionally as other bacteria.

Airway microbiota are associated with clinical outcomes

To determine the potential impact of vertebrate viruses on outcome, we compared virus enrichment differences in BAL samples across the three clinical outcome groups (≤28-days MV, >28-days MV, and deceased). As it pertains to the vertebrate RNA virome subfraction, there were significant differences (β diversity) between the three clinical outcome groups (**Supplementary Fig. 4**, PERMANOVA $p<0.01$).

Analysis of differential DNA virus abundance using DEseq did not show statistically significant differences. Because the virome includes viruses of bacteria and archaea, we also analyzed the phage data (including viruses of archaea). Phages impact the bacterial population—including bacterial pathogens—and so could be clinically relevant.

Oral commensals and poor clinical outcome

We also questioned the possible effects that antimicrobial exposure prior to sample collection might have on our sequence data. **Supplementary Table 11** shows beta diversity analysis evaluating the association between antimicrobial use prior to sample collection, and metagenome and metatranscriptome compositional data. PERMANOVA analysis of Bray Curtis dissimilarity show that only Amikacin use prior to sample collection

was found to be significantly associated with beta diversity of the metatranscriptome data while only Lopinavir/Ritonavir use prior to sample collection was associated with beta diversity differences in the metagenome data. Overall, most of the microbial signatures identified as enriched in the deceased or in subjects on prolonged MV are regular colonizers of healthy skin and mucosal surfaces rather than frequent respiratory pathogens.

Adaptive & innate immune responses to SARS-CoV-2

We evaluated whether levels of antibodies correlated with viral load in BAL samples. While viral load levels of SARS-CoV-2 measured with rRT-PCR did not correlate with BAL measurements of SARS-CoV-2 specific antibodies, sgRNA viral load levels negatively correlated with BAL levels of Anti-Spike (IgG and IgA), Anti-RBD (IgG and IgA) and the Neutralization assay (**Supplementary Table 12**). These data suggest that the IgG subfraction is an important marker of the adaptive immune response in the lung of critically ill COVID-19 patients and that both sub-fractions of IgG and IgA anti-SARS-CoV-2 may contribute to the viral replication control in the lower airways.

Upstream pathway prediction analyses of the host airway transcriptome support previously reported mitochondria dysfunction[1] (inhibition in mitochondrial related regulators NSUN3, MRPL14, MRPL12, LONP1, DAP3), and metabolic/gluconeogenesis dysregulation[2,3] (SIRT3) in critically ill COVID-19 subjects with poor outcome (**Supplementary Table 13**). We also observed decreased activation in the inflammatory response in critically ill COVID-19 subjects with poor outcome (phagocytes, neutrophils, and granulocytes, and leukocytes; Supplementary Table 10). A comparison of clinical outcome between the >28-days MV vs. ≤28-days MV groups showed upstream predicted inhibition in insulin, estrogen, beta-estradiol, EGF, EGFR, IL-5, and IL-10RA in the >28-days MV group (**Supplementary neTable 14**). These differences suggest that, at the stage that we sampled the lower airways of patients with critically COVID-19, an overt inflammatory tone was not predictive of worst outcome.

Cross-kingdom network analyses & SARS-CoV-2

Investigating module response on an individual gene level, Interleukin 4 induced 1 (IL4I1) appears as one of the most up-regulated genes in this module when comparing the deceased group with the ≤28-day MV group. The transporter 1, ATP binding cassette subfamily B member (TAP1) is also upregulated and a key regulator (hub gene). Together with *TAP2*, *TAP1* plays a central role in MHC I antigen presentation[4]. Transcriptional regulators *SP110* and *SP140*, both ISGs and also identified as hub genes, were down-regulated. Module 718 was also positively correlated with the SARS2-NWN (ρ = 0.31, P-value = 1.3e-3; enrichment FET P-value = 0.029, 3.7 FE of M178 by differentially expressed genes in deceased vs ≤28-days MV). The majority of genes in this module are down-regulated in the deceased group compared with the ≤28-day group. Some of the genes encode subunits of the mitochondrial ATP synthase, such as *ATP6* and *ATP8*, the cytochrome C oxidase, with *COX2* and *COX3* as well as the NADH dehydrogenase complex, such as *ND1-ND6*. *ND4L, ATP6, COX2, ND1, ND3, ND4L* and *ND6* are key regulators, potentially modulating the expression of the other genes in the module. These findings further support mitochondria dysfunction[1], potentially disrupting processes indicated by the module. Other down-regulated genes are humanin1 (*MTRNR2L1*) and R-spondin 1 (*RSPO1*). Humanin is known to protect against oxidative stress and mitochondrial dysfunction[5]. *RSPO1* protects against cell stress by activating the Wnt/β-catenin signaling pathway[6]. Non-coding RNAs, such as *MALAT1* and *RHOQ-AS*1 were found to be up-regulated. *MALAT1* is known to suppress IRF3-initiated antiviral innate immunity[7] while the function of *RHOQ-AS1* is unknown.

Supplementary Figure Legends:

Supplementary Figure 1. SARS-CoV-2 viral load in upper airway samples. Copies of the SARS-CoV-2 N gene per ml, normalized by the Human RNase P gene, in 142 upper airways comparing clinical outcome groups **(**Two-sided Mann–Whitney U p-value**)**.

Supplementary Figure 2. Topographical analyses of the bacterial load. Bacterial load measured by ddPCR targeting 16S rRNA gene in 23 background bronchoscope controls (BKG), 142 lower airway (BAL) and 142 upper airway (UA) samples.

Supplementary Figure 3. Average sequencing depth and genome coverage for SARS-CoV-2 and bacterial taxa identified as associated with poor outcome. (a) Average metatranscriptome sequencing read depth across sliding windows of 100 nt for SARS-CoV-2 and 10 kb for bacteria genomes. Column on the left shows data for samples with high read counts and column on the right shows data for samples with low read counts for the taxa of interest. **(b)** Average metagenome sequencing read depth across sliding windows of 10 kb for bacteria genomes. Column on the left shows data for samples with high read counts and column on the right shows data for samples with low read counts for the taxa of interest.

Supplementary Figure 4. Evaluation of associations between the lower airway RNA virome and clinical outcome. Comparisons between the three clinical outcome groups was performed for α diversity (Shannon Index, each dot denotes the Shannon diversity

of a sample while the box center depicts median, box inter-quartile range with median at the center and whiskers represent maximum and minimum value, left panel), β diversity (based on Bray Curtis Dissimilarity Index, right panel); Kruskal-Wallis p-value and PERMANOVA p-value respectively, across 5 background negative controls (bronchoscope), 118 bronchoalveolar lavage (BAL) and 64 upper airway (UA) samples.

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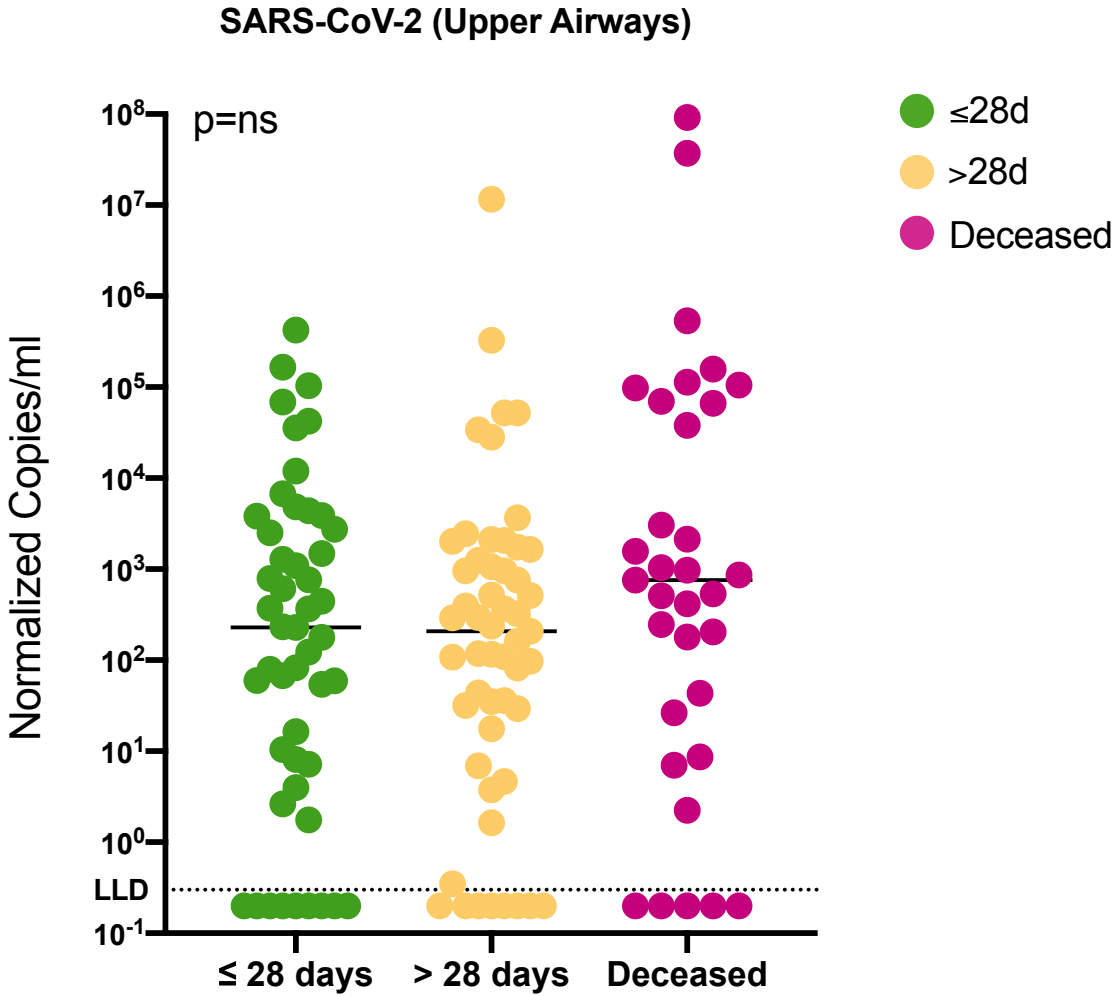
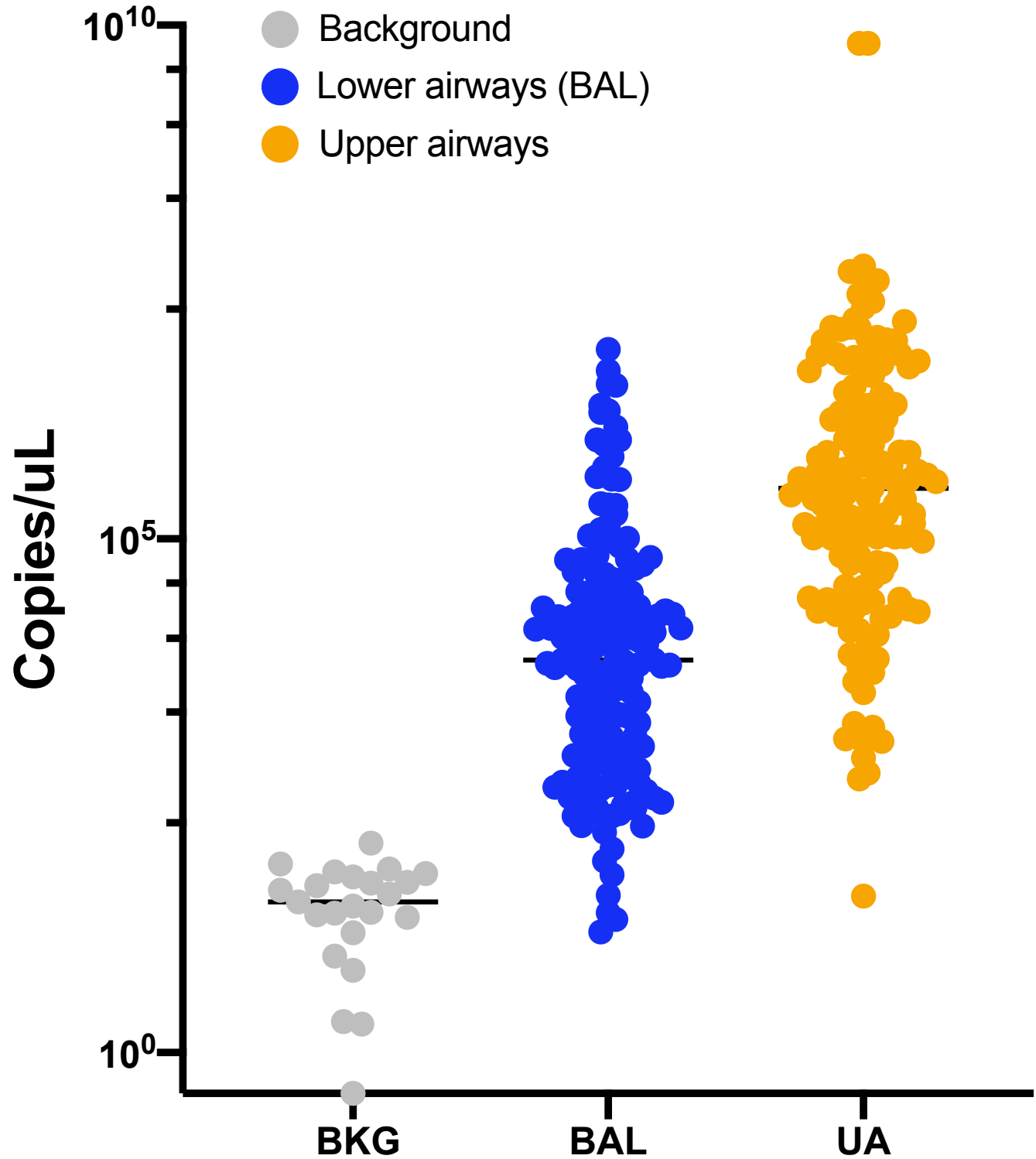
Supplementary Figure 1



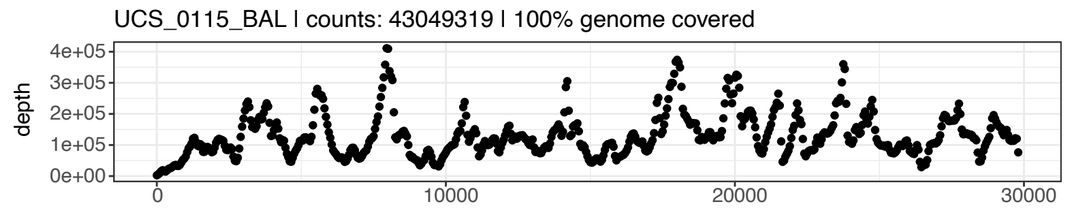
Supplementary Figure 2

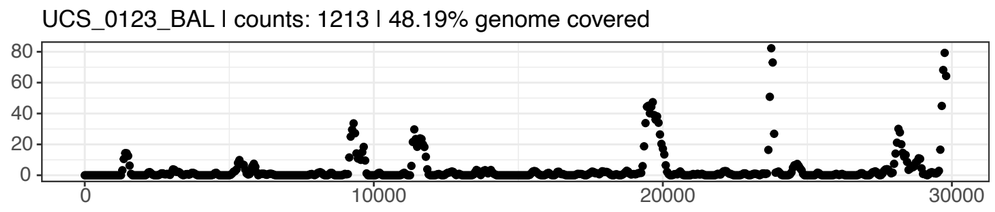


Supplementary Figure 3

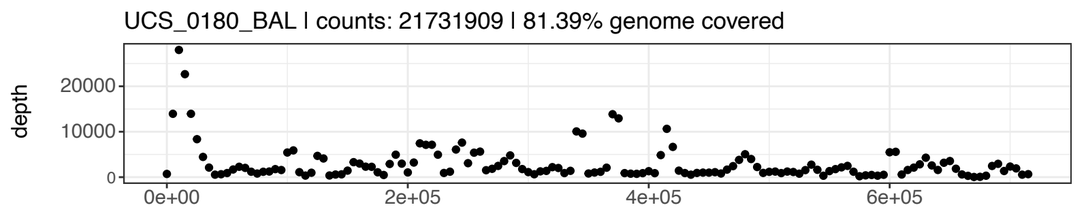
(a) Metatranscriptome

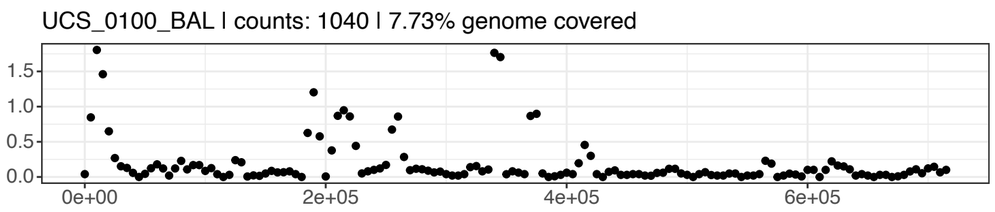
SARS-CoV-2



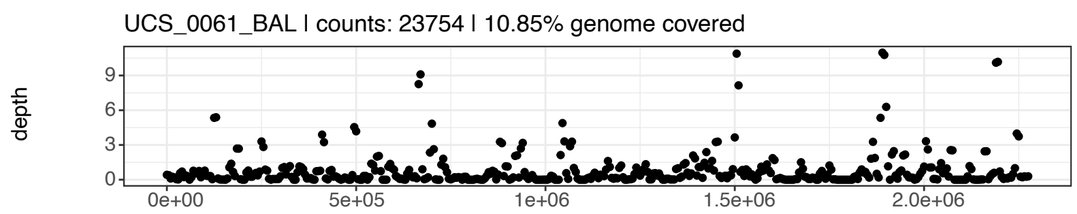


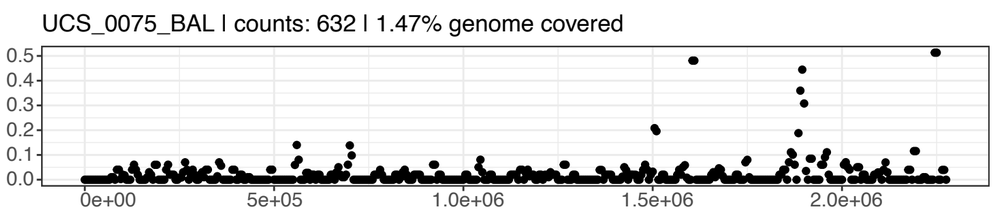
Mycoplasma salivarium



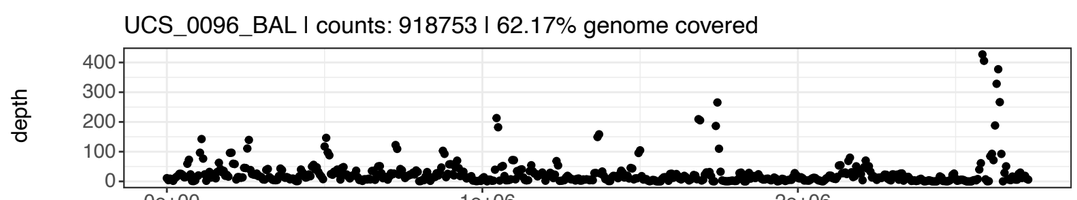


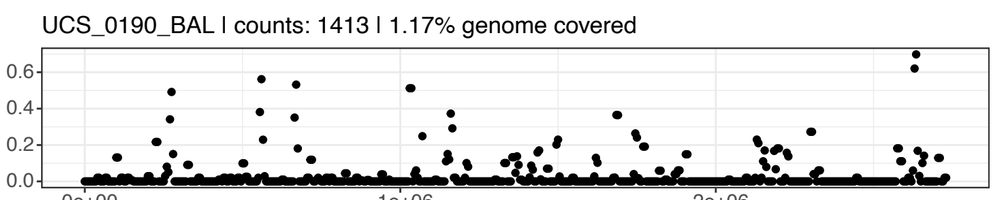
Bifidobacterium breve



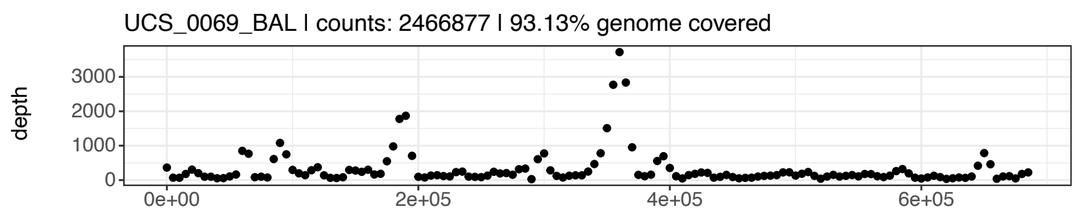


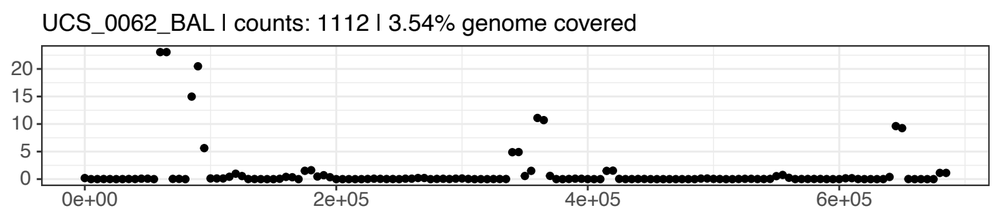
Lacticaseibacillus rhamnosus



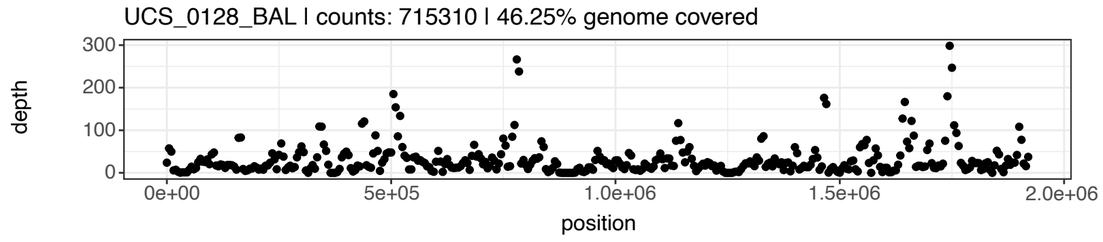


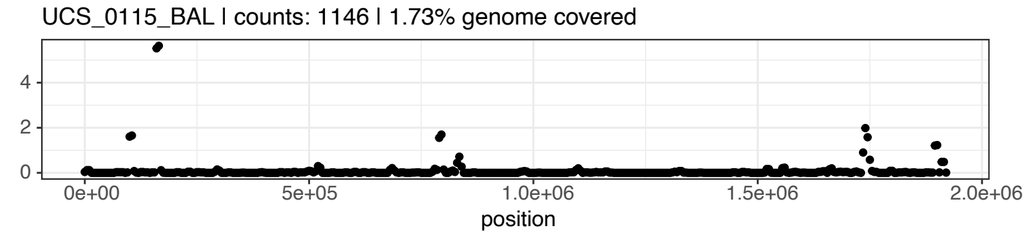
Mycoplasma hominis





Streptococcus oralis





(b) Metagenome

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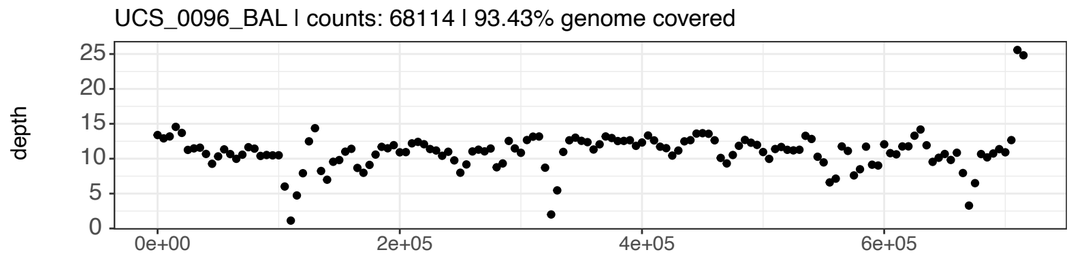

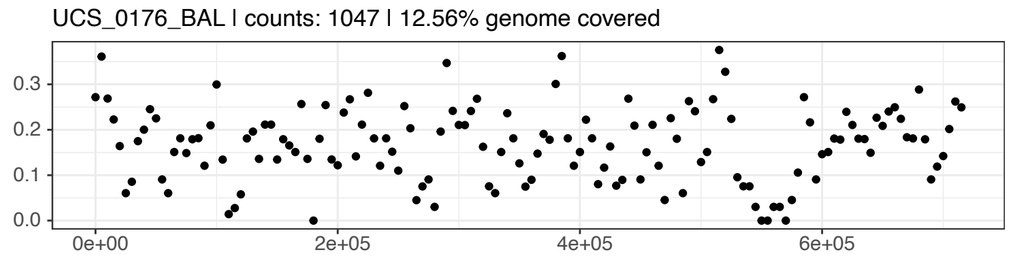

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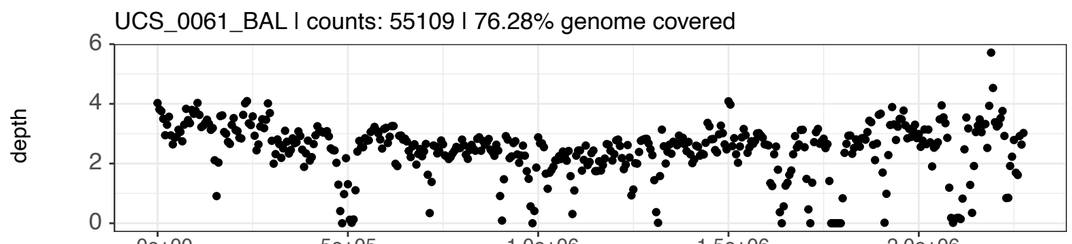

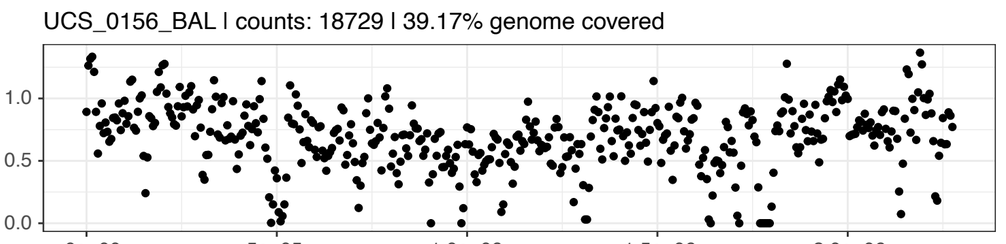

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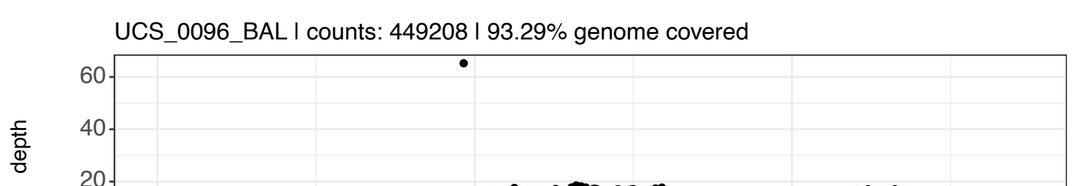

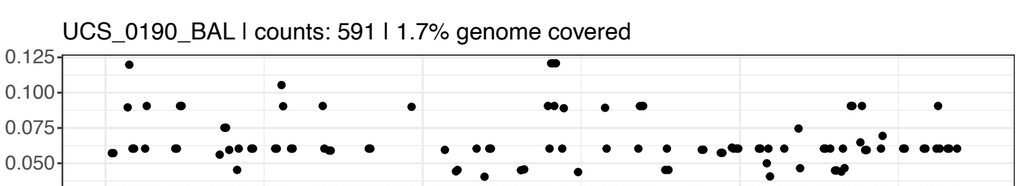

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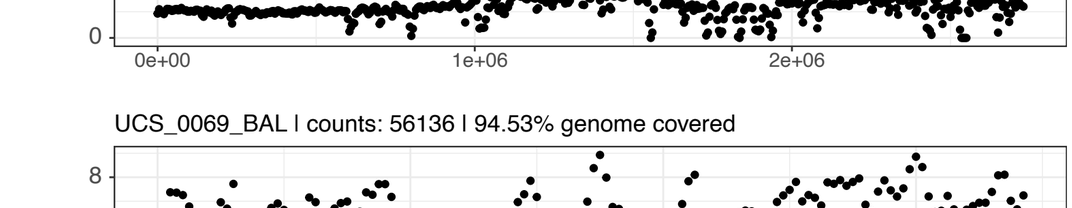

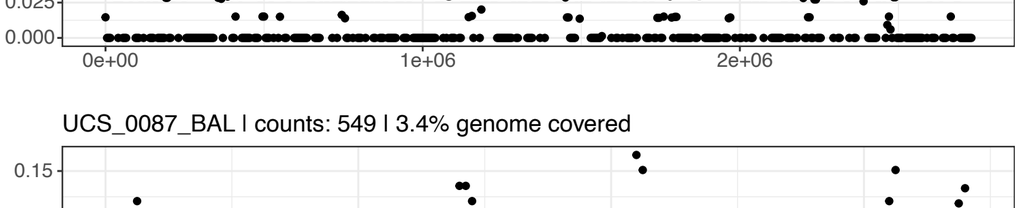

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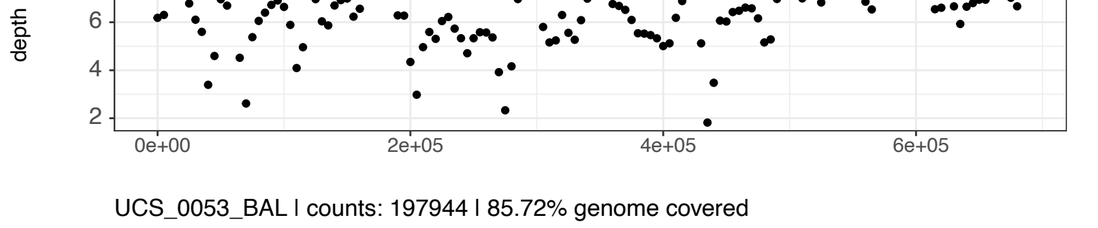

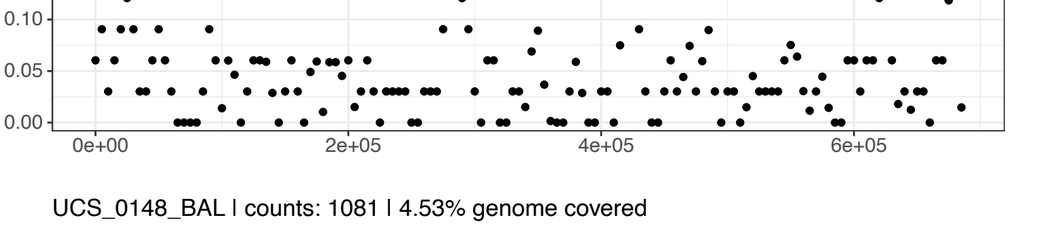

Supplementary Figure 4