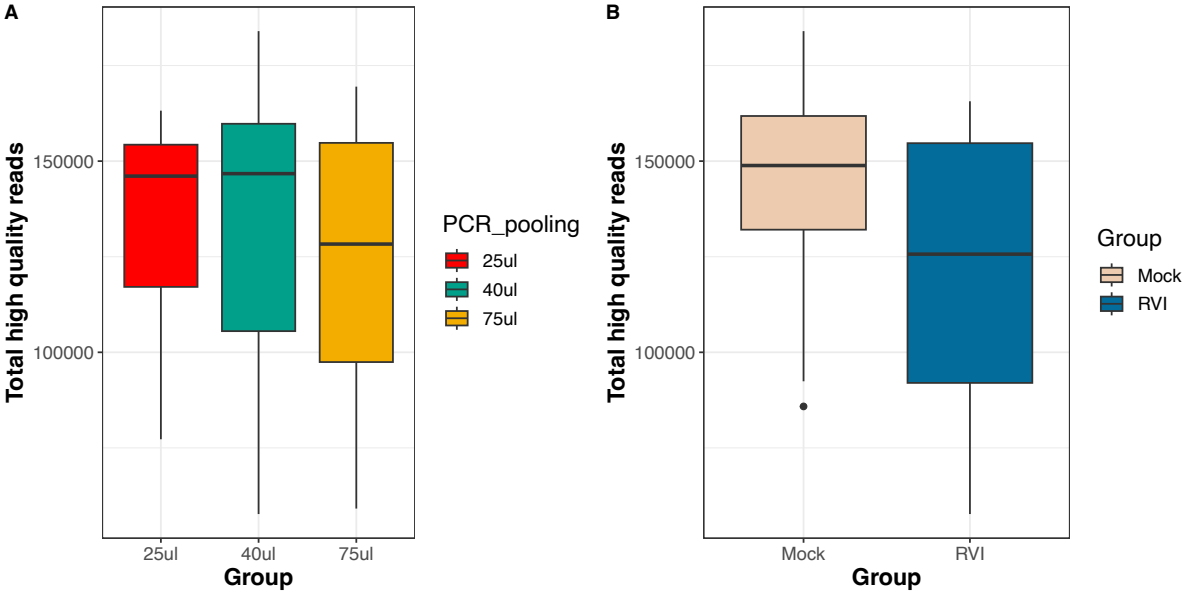
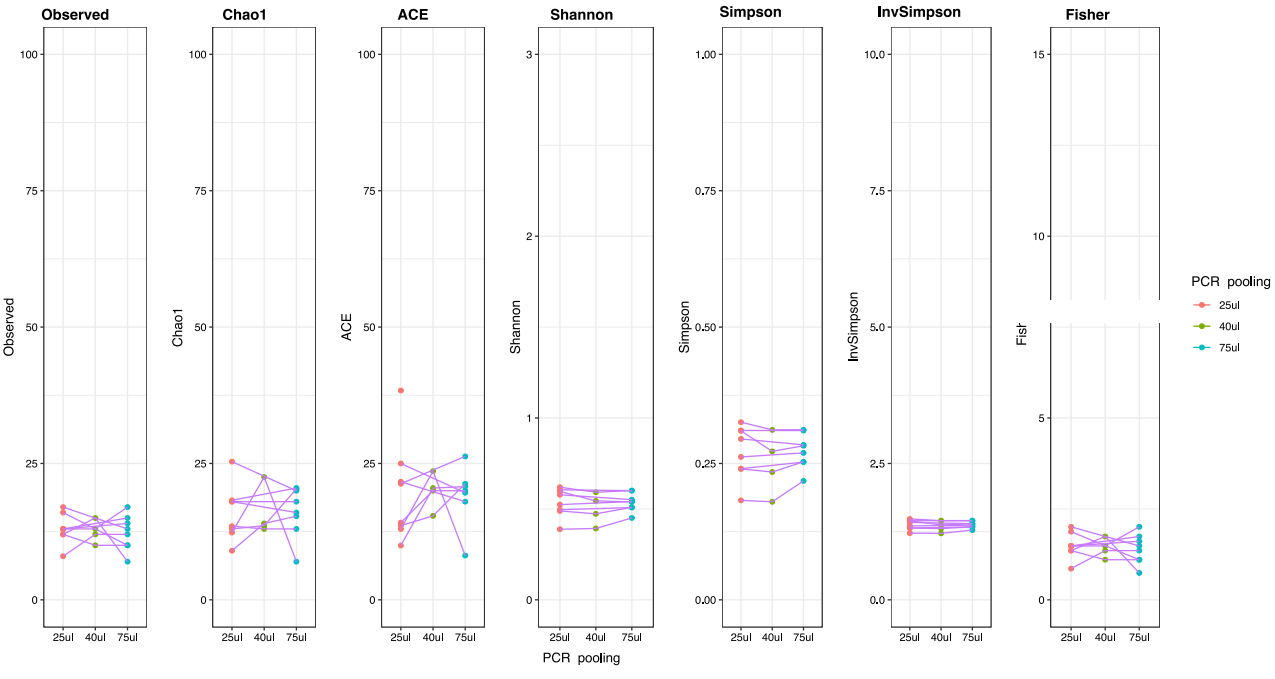
Supplementary Materials

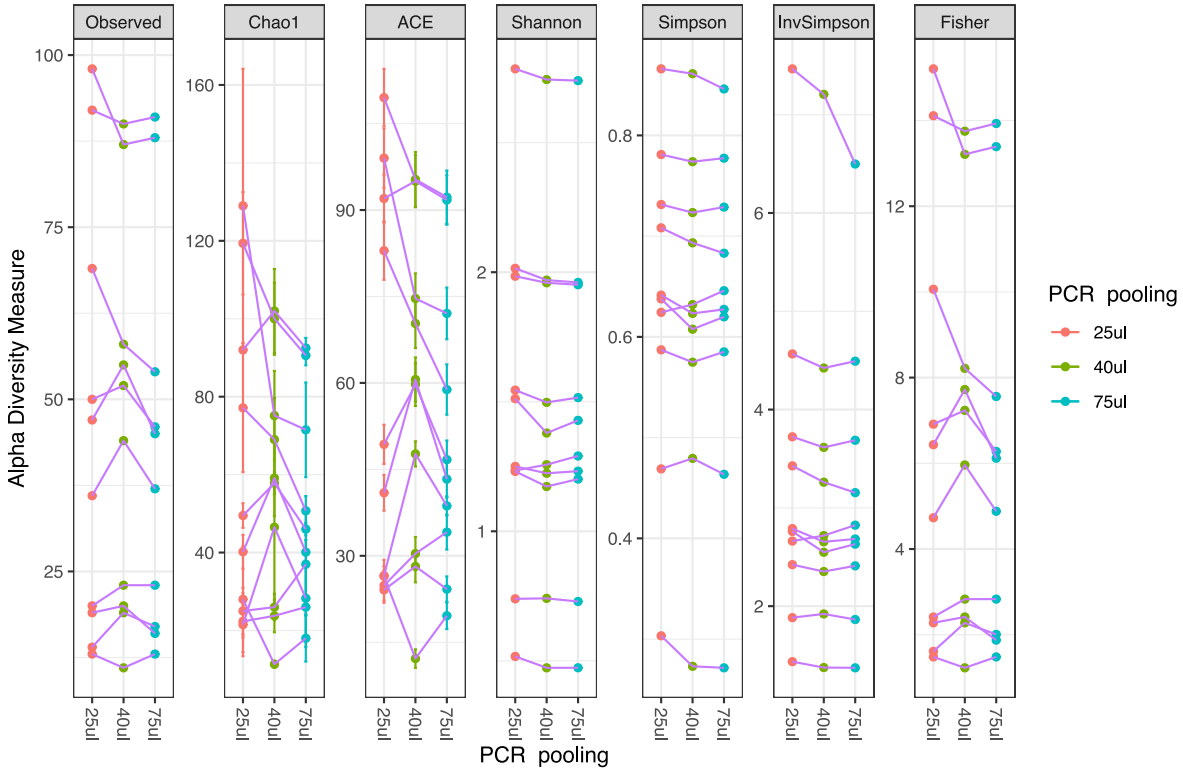
Supplementary Figures



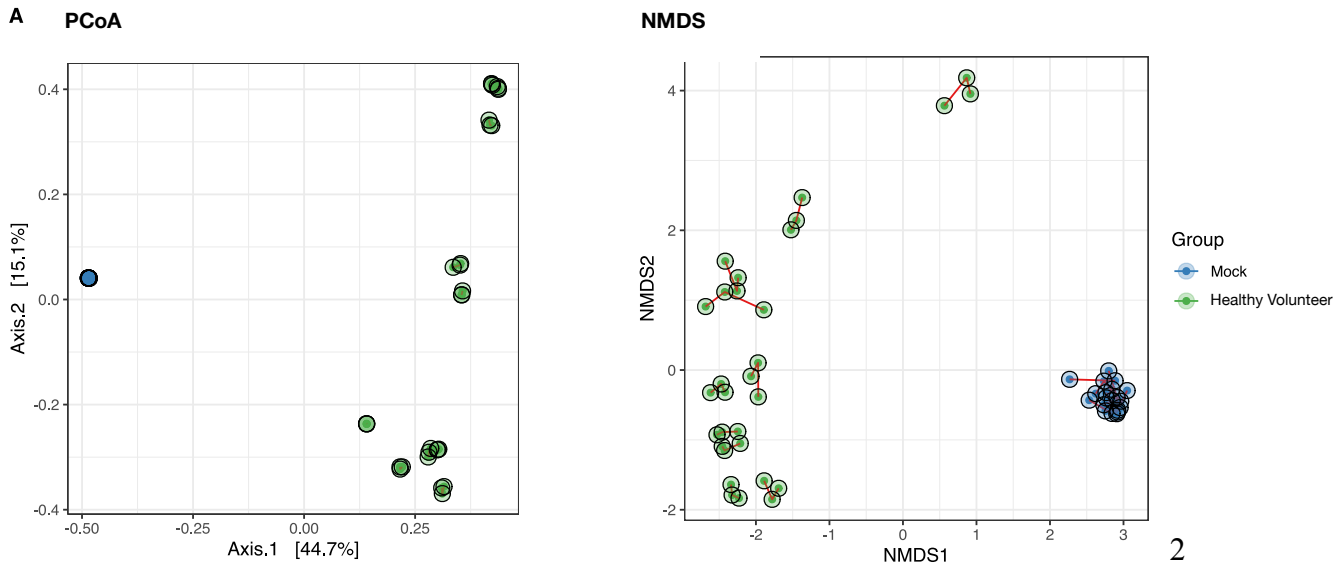
Supplementary Figure 1: Boxplot of high-quality reads post mothur qc. **(A)** Comparing PCR reaction replicates conducted in triplicate at 25µl (red), in duplicate at 40µl (green), and as a single 75µl reaction (yellow), from healthy nasal swabs. **(B)** Comparing healthy nasal swabs (RVI, Respiratory Virus and Microbiome Initiative) (blue) with mock community experiments (beige). Data presented are from Experiment 1.



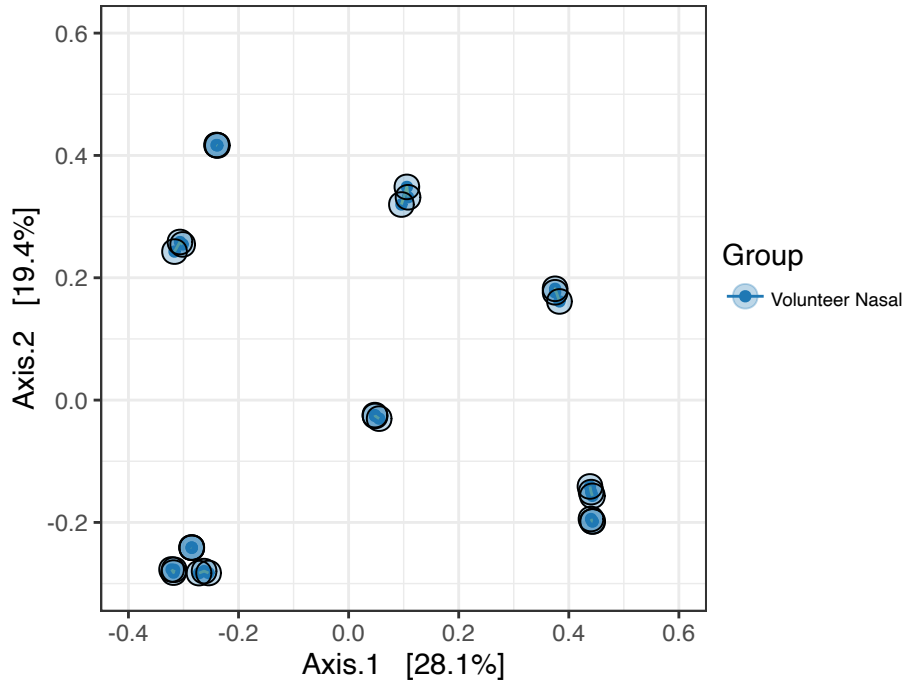
Supplementary Figure 2: Alpha diversity by multiple indices of mock community replicates. Plot comparing PCR reaction conducted in triplicate (25μl), duplicate (40μl), and as a single reaction (75μl). Replicates by type of mastermix preparation - manual (green) and premixed (blue) are linked by a red-line. Alpha diversity is calculated after rarefication of high-quality reads. Note, y-axis is adjusted to reflect alpha indices variation seen amongst nasal samples (Supplementary Figures 4, 5, and 15). Data presented are from Experiment 1.



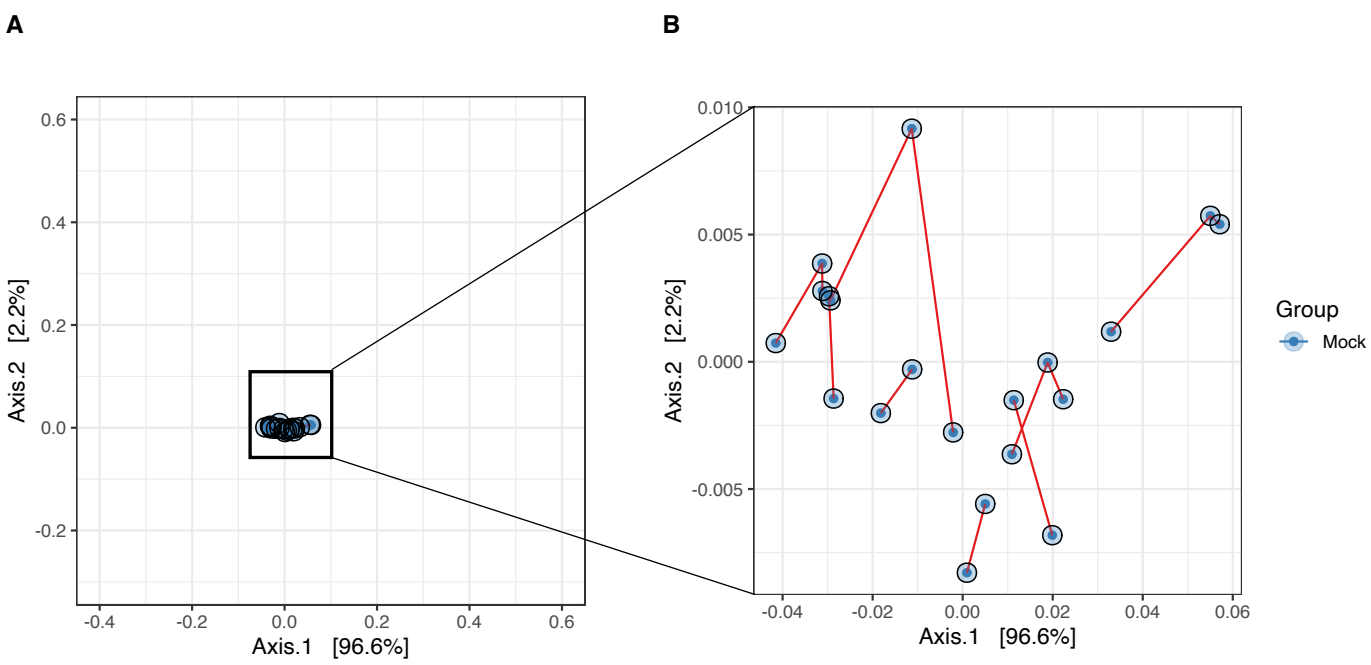
Supplementary Figure 3: Alpha diversity by multiple indices post mothur qc of healthy nasal swabs obtained from Healthy Volunteers at the Wellcome Sanger Institute. Plot comparing PCR reaction replicates conducted in triplicate at 25μl (red), duplicate at 40μl (green), and as a single 75μl reaction (blue), linked by purple-line. Alpha diversity is calculated after rarefication of high-quality reads. Data presented are from Experiment 1.



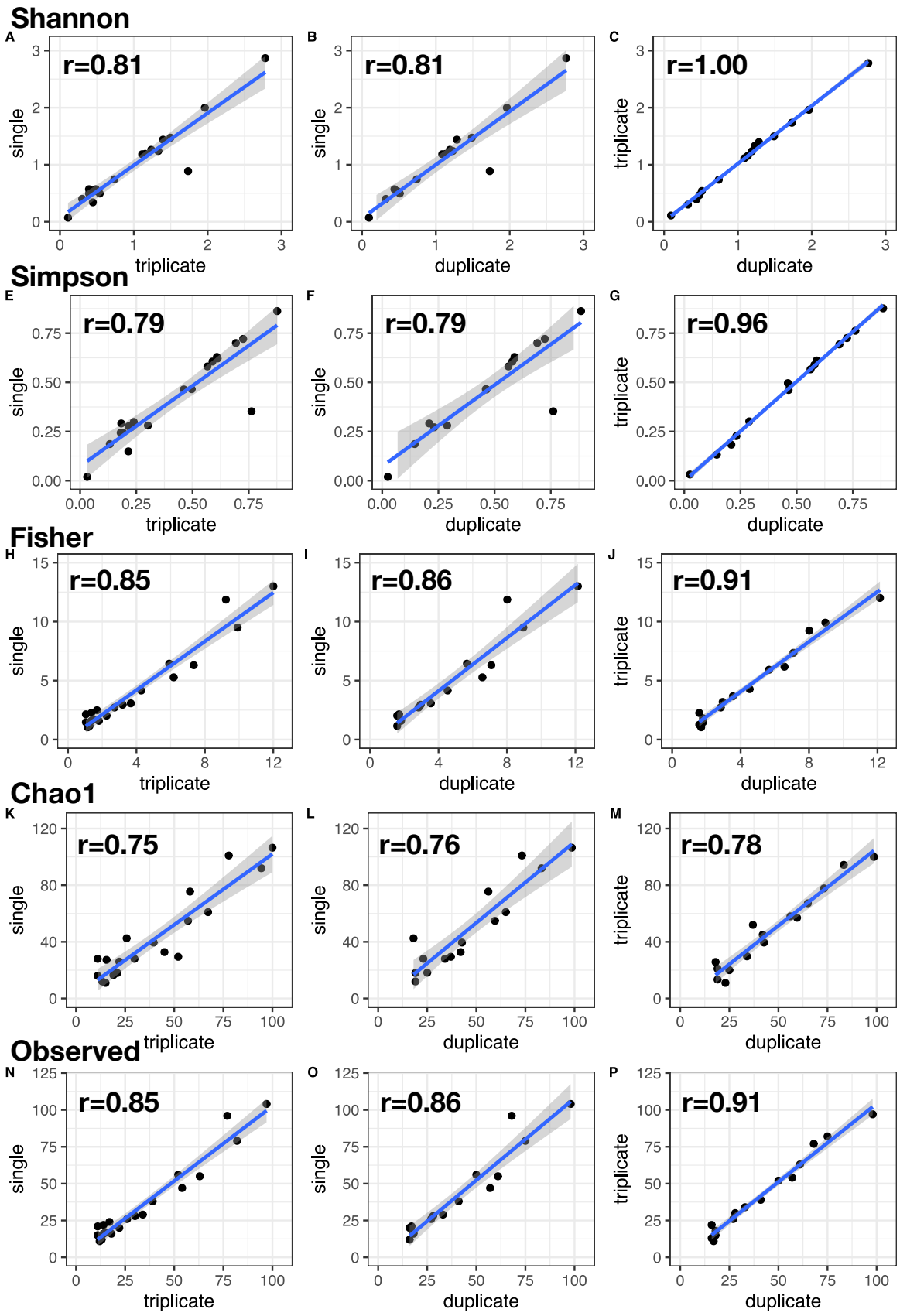
Supplementary Figure 4: Ordination plots of Bray-Curtis dissimilarity indices between replicate samples from different PCR pools. Principle Component Analysis (PCoA) (**A**) and Non-metric multidimensional scaling (NMDS) (**B**) of Bray-Curtis dissimilarity indices. Nasal samples obtained from healthy participants at the Wellcome Sanger Institute (green) and mock community isolates (blue) are represented. Replicates from different PCR pools are linked by a red-line. Replicates by various PCR pooling strategies cluster (no significant difference by PERMANOVA analysis, p=0.99), whereas the mock community samples are clearly distinct from nasal samples (significant difference by PERMANOVA analysis, p=0.001). In the PCoA plot, replicates cluster very closely, such that the red 'group' line is not visible. Data presented are from Experiment 1.



Supplementary Figure 5: Principle Component Analysis of Bray-Curtis dissimilarity indices between replicate samples from PCR pooling strategies (linked by green line) from nasal samples of healthy volunteers via the Wellcome Sanger Institute (blue). Replicates from libraries with different PCR pooling strategies are nearly indistinguishable. High-quality reads from the Operational Taxonomic Unit Matrix are rarefied and then converted to percentage abundance for each sample. Data presented are from Experiment 1.

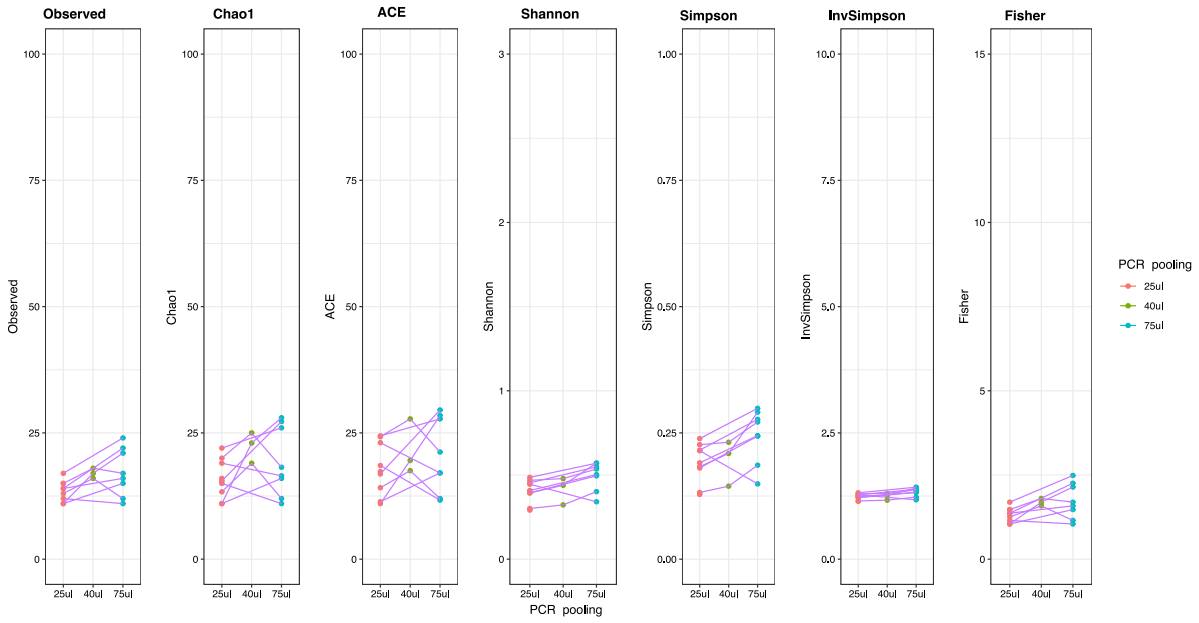


Supplementary Figure 6: Principle Component Analysis of Bray-Curtis dissimilarity indices between replicate samples from PCR pooling strategies (linked by red line) from mock community serially diluted preparations. **(A)** Axis of PCoA is consistent with the PCoA plot in Supplementary Figure 8 and the mock community samples are observed to closely cluster. **(B)** Axis of PCoA is exaggerated to allow closer examination of the mock community cluster. High-quality reads from the Operational Taxonomic Unit Matrix in **A** and **B** are rarefied and then converted to percentage abundance in each sample. Data presented are from Experiment 1.

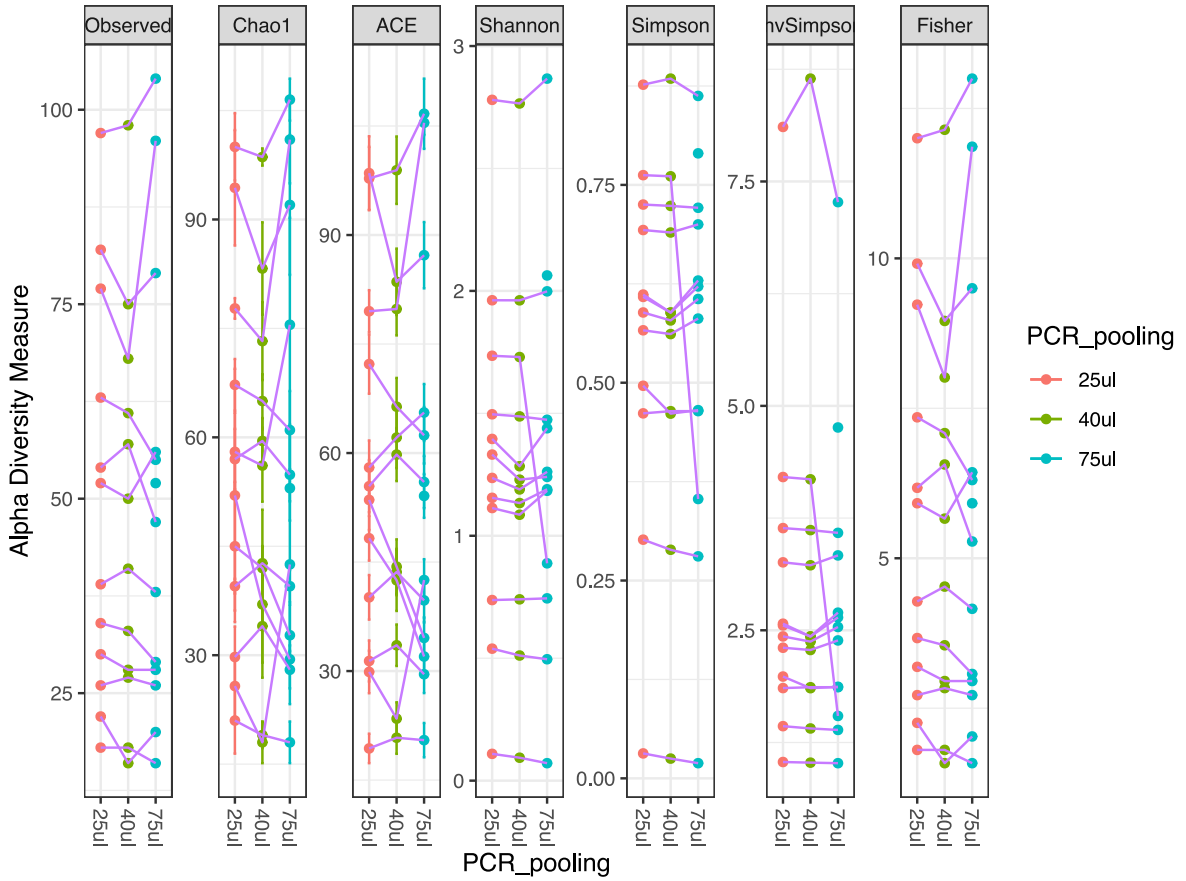


Supplementary Figure 7: Correlation of alpha diversity indices by PCR pooling strategy, after rarefication of high-quality sample reads with controls removed. Replicates are from healthy volunteer nasal samples from the Wellcome Sanger Institute and the serially diluted mock microbial community. Alpha indices represented include Shannon, Simpson, Fisher, Chao1,

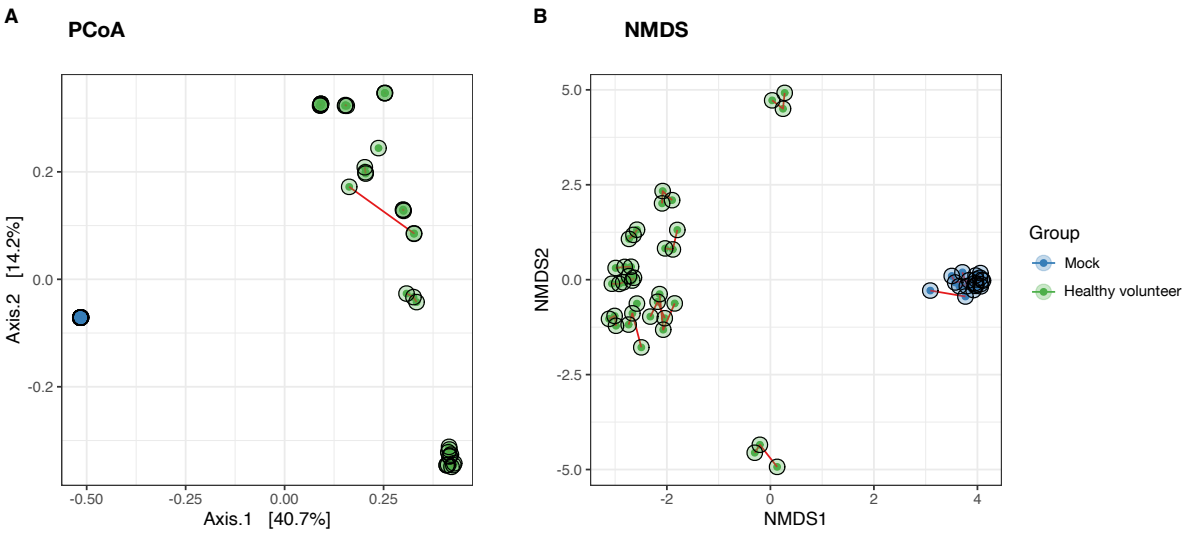
and observed richness (**A-P**). Pairwise Kendall's rank correlation coefficient (**r**) is presented in the top-left of each plot. A strong correlation between PCR pools is observed by all alpha indices. A linear regression model is fitted to the observed values. Data presented are from Experiment 2.



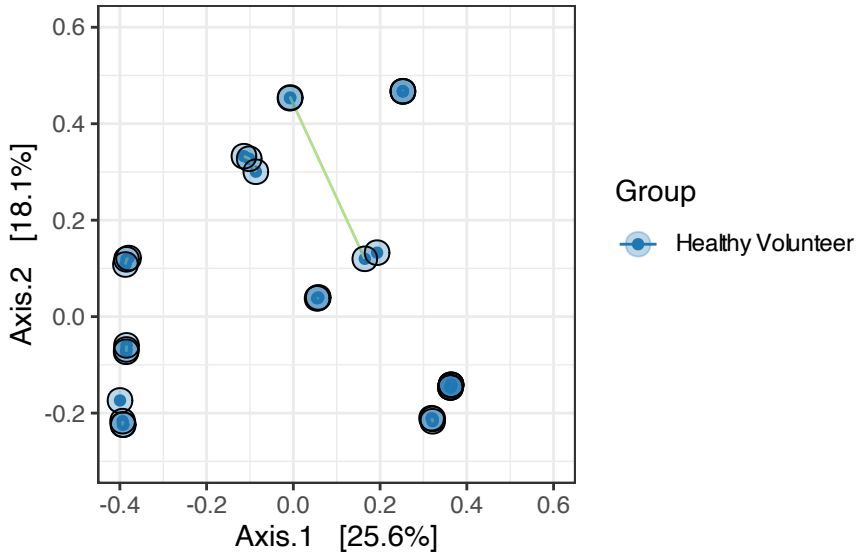
Supplementary Figure 8 Alpha diversity by multiple indices of mock community replicates. Plot comparing PCR reaction conducted in triplicate (25μl), duplicate (40μl), and as a single reaction (75μl). Replicates by type of mastermix preparation - manual mix (red) and premixed (green) are linked by a blue-line. Alpha diversity is calculated after rarefication of high-quality reads. Note, y-axis is adjusted to reflect alpha indices variation seen amongst nasal samples (Supplementary Figures 4, 5, and 15). Data presented are from Experiment 2.



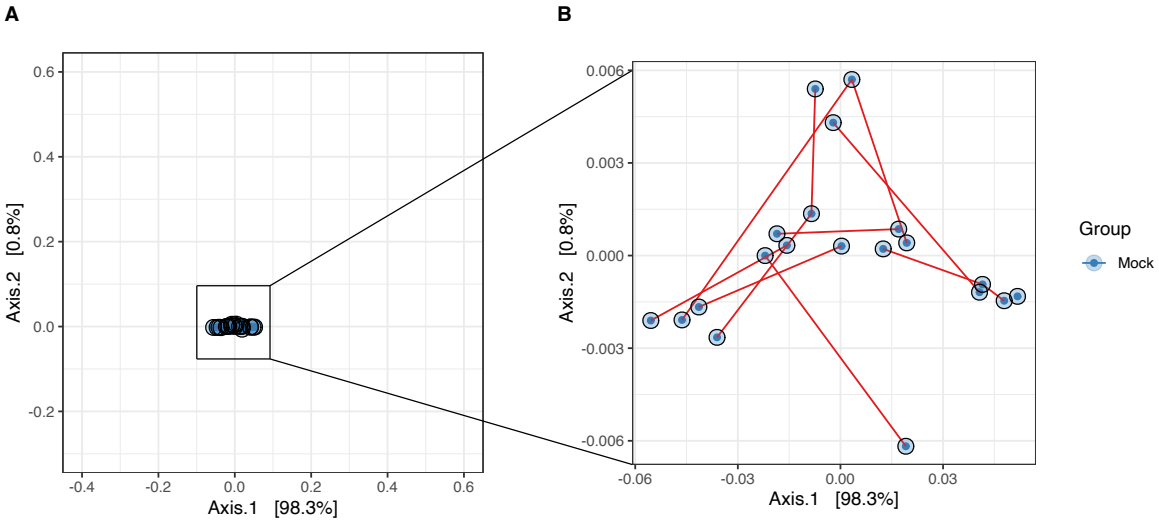
Supplementary Figure 9: Alpha diversity by multiple indices post mothur qc of healthy nasal swabs obtained from the Healthy Volunteers at the Wellcome Sanger Institute. Plot comparing PCR reaction replicates conducted in triplicate at 25µl (red), duplicate at 40µl (green), and as a single 75µl reaction (blue), replicates linked by purple-line. Alpha diversity is calculated after rarefication of high-quality reads. Data presented are from Experiment 2.



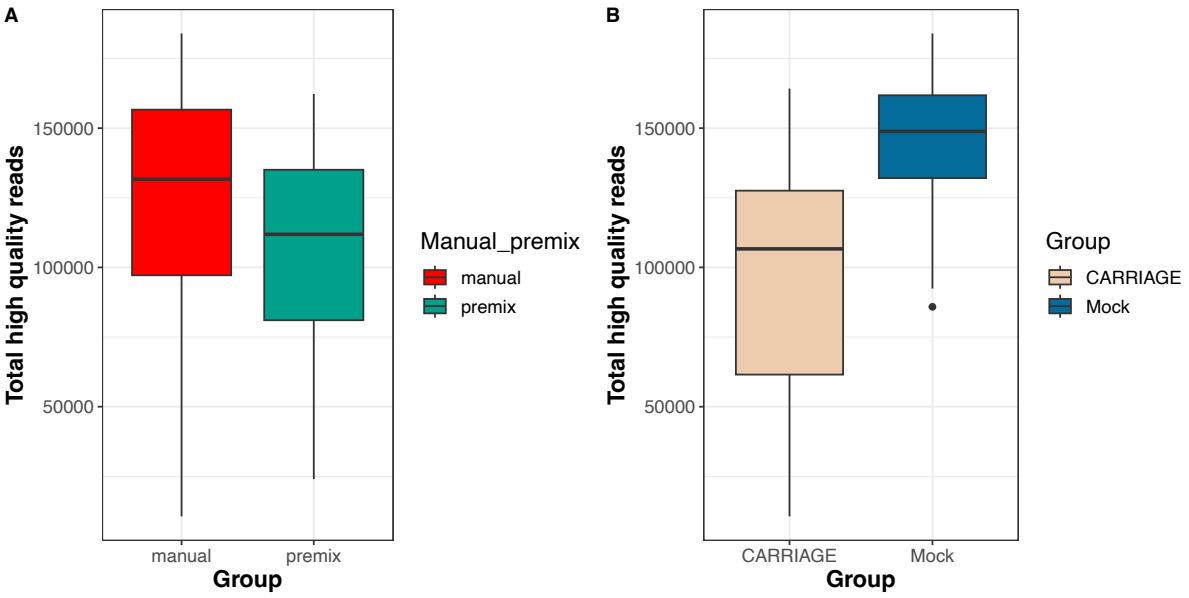
Supplementary Figure 10: Ordination plots of Bray-Curtis dissimilarity indices between replicate samples from different PCR pools. Principle Component Analysis (PCoA) (**A**) and Non-metric multidimensional scaling (NMDS) (**B**) of Bray-Curtis dissimilarity indices. Nasal samples from healthy volunteer obtained via the Wellcome Sanger Intitute(green) and mock community isolates (blue) are represented. Replicates from different PCR pools are linked by a red-line. Replicates from different PCR pooling strategies cluster (no significant difference by PERMANOVA analysis, p=0.94), whereas the mock community samples are clearly distinct from nasal samples (significant difference by PERMANOVA analysis, p<0.001). In the PCoA plot, replicates cluster very close, such that the red line connecting them is not visible. Data presented are from Experiment 2.



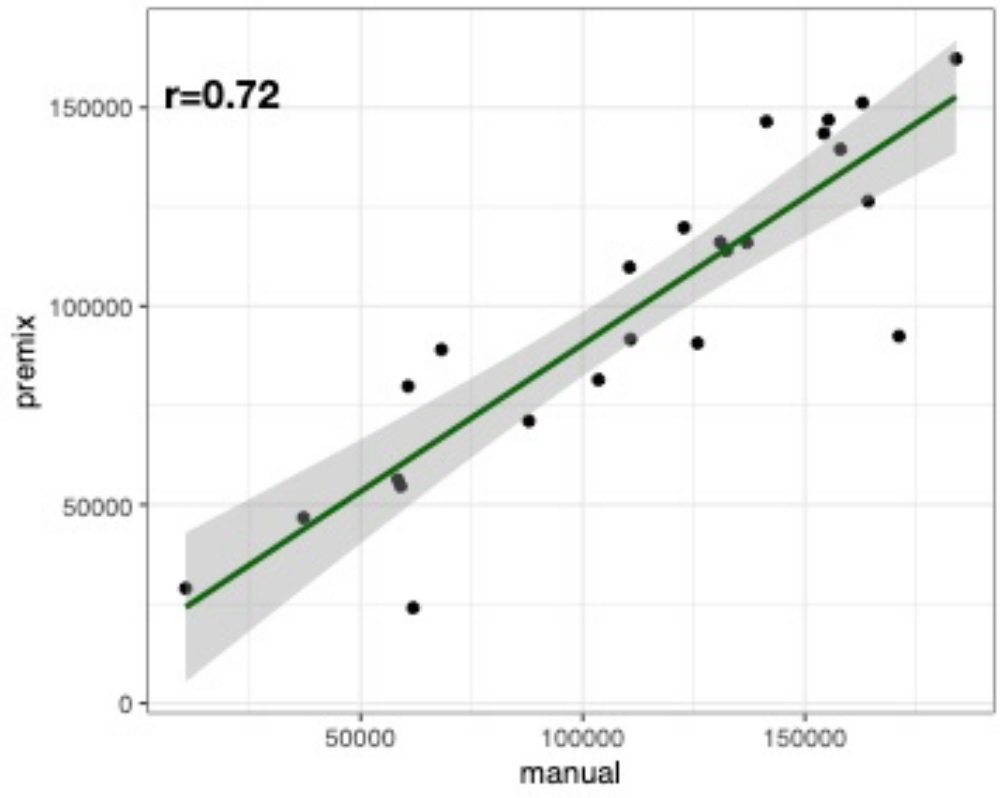
Supplementary Figure 11: Principle Component Analysis of Bray-Curtis dissimilarity indices between replicate samples from PCR pooling strategies (linked by green line) from nasal samples of healthy volunteers obtained via the Wellcome Sanger Institute (blue). Replicates from libraries with different PCR pooling strategies are nearly indistinguishable. High-quality reads from the Operational Taxonomic Unit Matrix are rarefied and then converted to percentage abundance in each sample. Data presented are from Experiment 2.



Supplementary Figure 12: Principle Component Analysis of Bray-Curtis dissimilarity indices between replicate samples from PCR pooling strategies (linked by red line) from mock community serially diluted preparations. **(A)** Axis of PCoA is consistent with the PCoA plot in Supplementary Figure 11and the mock community samples are observed to closely cluster. **(B)** Axis of PCoA is exaggerated to allow closer examination of the mock community cluster. High-quality reads from the Operational Taxonomic Unit Matrix in **A** and **B** are rarefied and then converted to percentage abundance in each sample. Data presented are from Experiment 2.



Supplementary Figure 13: Boxplot of high-quality reads post mothur qc. **(A)** Comparing manual (red) and premixed (green) mastermix replicates from healthy nasal swabs (CARRIAGE study). **(B)** Comparing healthy nasal swabs (CARRIAGE) (beige) with mock community experiments (blue). Data presented are from Experiment 1.



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168 **Supplementary Figure 14.** Correlation of high-quality reads obtained from samples by
169 comparing mastermix preparations (premixed vs manual). Replicates are from CARRIAGE
170 nasal samples from the Wellcome Sanger Institute and the serially diluted mock microbial
171 community. Pairwise Kendall's rank correlation coefficient (r) is presented in the top-left of the
172 plot. A linear regression model is fitted to the observed values. Data presented are from
173 Experiment 1.

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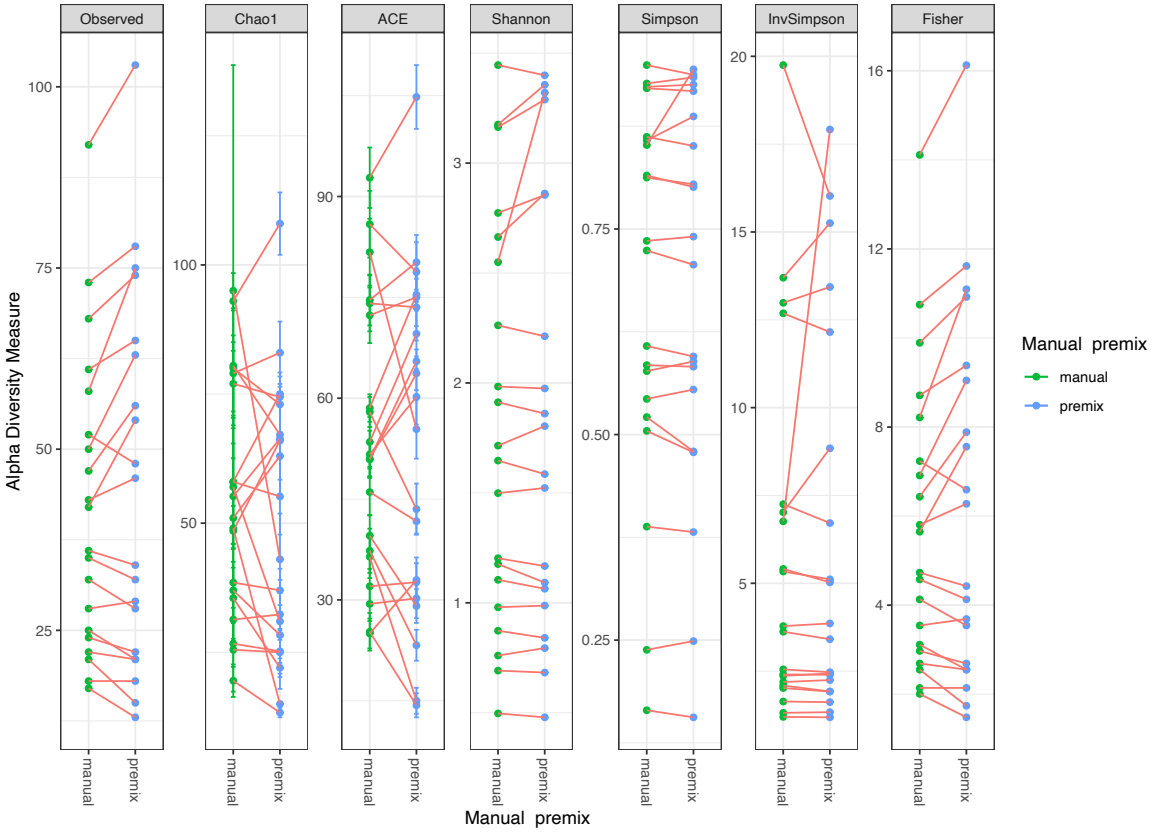
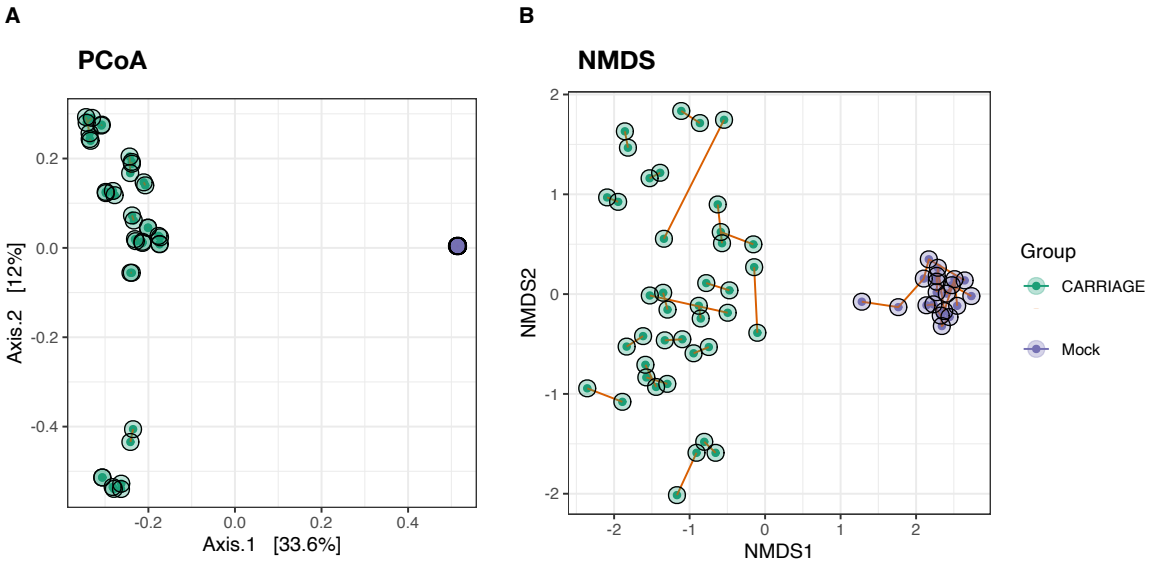
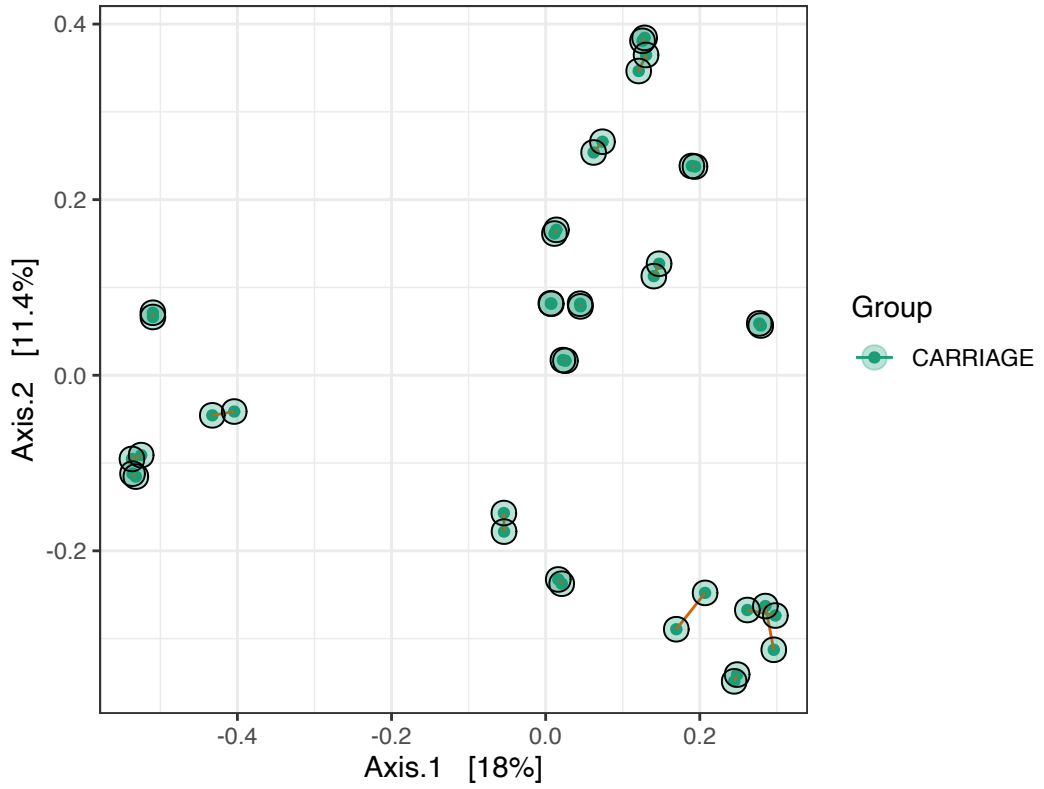


Supplementary Figure 15: Alpha diversity by multiple indices post mothur qc of healthy nasal swabs from the CARRIAGE study, comparing manual (green) and premixed (blue) mastermix replicates (linked by red-line). Alpha diversity is calculated after rarefication of high-quality reads. Data presented are from Experiment 1.

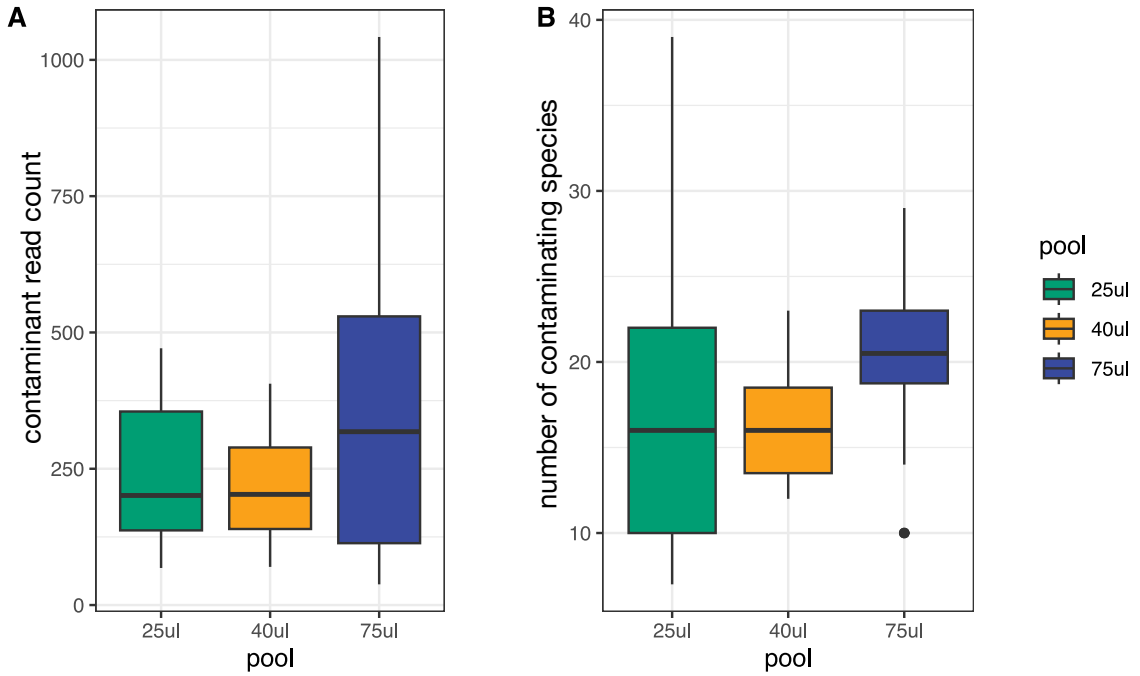


Supplementary Figure 16: Ordination plots of Bray-Curtis dissimilarity indices between replicate samples from different mastermix preparations. Principle Component Analysis (PCoA) (**A**) and Non-metric multidimensional scaling (NMDS) (**B**) of Bray-Curtis dissimilarity indices. Nasal samples from healthy participants (CARRIAGE) (green) and mock community

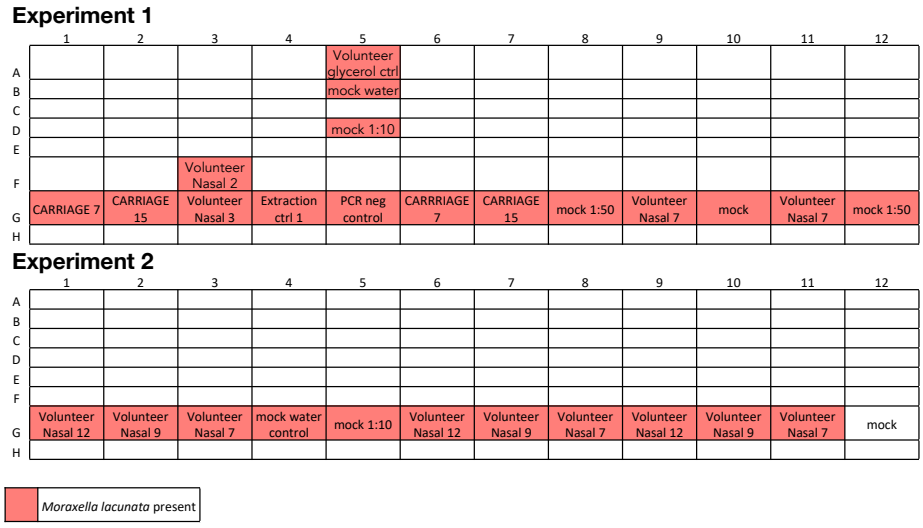
isolates (purple) are represented. Replicates from different mastermix preparations are linked by a red-line. Replicates from individuals with different mastermix preparations cluster (no significant difference by PERMANOVA analysis, p=1), whereas the mock community samples are clearly distinct from nasal samples (significant difference by PERMANOVA analysis, p=0.001). In the PCoA plot, replicates cluster very close, such that the red line connecting them is not visible. Data presented are from Experiment 1.



Supplementary Figure 17: Principle Component Analysis of Bray-Curtis dissimilarity indices between replicate samples with PCR using manually prepared or premixed mastermix (linked by red line) from healthy nasal samples of CARRIAGE participants (green). Replicates from libraries with different mastermixes used are nearly indistinguishable. High-quality reads from the Operational Taxonomic Unit Matrix are rarefied and then converted to percentage abundance in each sample. Data presented are from Experiment 1.



Supplementary Figure 18: (A) Boxplot of high-quality contaminant reads in the mock microbial community samples by pooling strategy - in triplicate at 25µl (red), in duplicate at 40µl (green), and as a single 75µl reaction (yellow). **(B)** Boxplot of number of contaminating species in the mock microbial community samples by pooling strategy - in triplicate at 25µl (red), in duplicate at 40µl (green), and as a single 75µl reaction (yellow).



Supplementary Figure 19: Distribution of *Moraxella lacunata* on plate map of Experiment 1 and Experiment 2. Contamination of *Moraxella lacunata* is seen in Row G on the plate maps of both experiments demonstrating a batch effect related to primer stock contamination. 1 or 2 reads mapping to *Moraxella lacunata* are seen in samples outside of Row G in plate 1 and none in plate 2.

Supplementary Tables

Supplementary Table 1: Differences in alpha diversity between PCR pooling strategies using Kruskall-Wallis tests. No significant difference is seen by Shannon, Simpson, Fisher, Chao1, and Observed indices between PCR pool strategies (chi-squared and p-values presented to 2 d.p.). Alpha diversity is calculated after rarefication of high-quality reads.

EXPERIMENT 1		
ALPHA INDEX	**chi-squared**	**p-value**
SHANNON	**0.15**	**0.93**
SIMPSON	**0.23**	**0.89**
FISHER	**0.68**	**0.71**
CHAO1	**0.56**	**0.76**
OBSERVED	**0.68**	**0.71**
EXPERIMENT 2		
ALPHA INDEX		
SHANNON	**0.15**	**0.93**
SIMPSON	**0.23**	**0.89**
FISHER	**0.68**	**0.71**
CHAO1	**0.56**	**0.76**
OBSERVED	**0.68**	**0.71**

Supplementary Table 2: Relative abundance of mock community by PCR pool strategy compared to manufacturer reported. Data presented are from Experiment 1.

Sample	Listeria monocytogenes	Pseudomonas aeruginosa	Bacillus subtilis	Salmonella enterica	Escherichia coli	Lactobacillus fermentum	Staphylococcus aureus	Enterococcus faecalis
Mock	80.5546932	16.393107	2.28931499	0.39498279	0.33685824	0.02708076	0.00066051	0.00330253
Mock_40ul	81.8466226	15.2278354	2.27046438	0.33297968	0.29489377	0.02339563	0	0.00380859
Mock_75ul	81.4774762	15.5158048	2.31354265	0.37111233	0.2954716	0.02363773	0.00118189	0.00177283
Mock_premix	81.6446133	14.9945722	2.53124633	0.44595705	0.36307358	0.01466964	0.00513437	0.00073348
Mock_premix_75ul	82.0907493	14.6272248	2.52691323	0.38249175	0.33313797	0.03022919	0.00616922	0.00308461
Mock 1:10	82.1439959	14.5661062	2.41906341	0.45490296	0.37837723	0.03684572	0	0.00070857
Mock 1:10_40ul	84.2815218	12.7867764	2.22404961	0.39909167	0.27612013	0.03168592	0	0.00075443
Mock 1:10_75ul	83.5050724	14.0669732	1.74465581	0.34913066	0.30424244	0.02793045	0.00099752	0.00099752
Mock 1:10_premix	82.6928655	13.9633643	2.57401885	0.39937825	0.34359916	0.02231163	0.00297488	0.00148744
Mock 1:10_premix_75ul	83.4823472	13.2637101	2.5231034	0.40435225	0.32033987	0	0.00478119	0.00136605
Mock 1:50	86.6069345	10.8782962	1.94057898	0.2867881	0.2530122	0.02947715	0.00184232	0.00307054
Mock 1:50_40ul	86.9211273	10.4881271	2.04466018	0.30464489	0.21426269	0.02085743	0.00316022	0.00316022
Mock 1:50_75ul	85.4585188	11.7674007	2.12590772	0.30396642	0.29715658	0.03962088	0.00185723	0.00557169
Mock 1:50_premix	85.1549517	11.8845018	2.31812027	0.33467717	0.27312735	0.02692805	0.00769373	0
Mock 1:50_premix_75ul	84.7011715	12.2483288	2.33437024	0.3878483	0.28195115	0.037064	0.00794229	0.00132371
Mock1:100	89.8535779	8.09484769	1.68840153	0.155005	0.16525326	0.03843099	0.00448362	0
Mock1:100_40ul	89.5793153	8.27868757	1.76327571	0.18819184	0.14494278	0.03798904	0.00759781	0
Mock1:100_75ul	87.9312632	9.71117753	1.90259168	0.26299155	0.19197603	0	0	0
Mock1:100_premix	86.2931075	10.8976154	2.3099538	0.28932755	0.20532923	0.00116664	0	0.00349993
Mock1:100_premix_75ul	86.4103008	11.0253189	1.96551948	0.29022547	0.27506443	0.03140499	0	0.00216586
Actual	**0.959**	**0.028**	**0.012**	**0.0007**	**0.00069**	0.00012	**0.000001**	**0.000007**

Supplementary Table 3: Differences in Alpha diversity between mastermix choice using Mann-Whitney-U tests. No significant difference is seen by Shannon, Simpson, Fisher, Chao1, and Observed indices between manually prepared and premixed mastermix. Alpha diversity is calculated after rarefication of high-quality reads.

EXPERIMENT 1	
ALPHA INDEX	**p-value**
SHANNON	**0.42**
SIMPSON	**0.50**
FISHER	**0.31**
CHAO1	**0.40**
OBSERVED	**0.31**
EXPERIMENT 2	**p-value**
ALPHA INDEX	
SHANNON	**0.79**
SIMPSON	**0.85**
FISHER	**0.49**
CHAO1	**0.56**
OBSERVED	**0.49**

Supplementary Methods and Results

Original Protocol

The manually prepared master mix contained 5ul 5x Q5 Buffer (NEB, USA), 0.5ul 10mM dNTPs (NEB, USA), 0.25ul Taq polymerase (NEB, USA), 14.25ul nuclease free water (ThermoFisher Scientific, USA), 1.25ul of both the forward and reverse indexed cartridge purified primers (ThermoFisher Scientific, USA) diluted to10uM in nuclease free water as before. Primer sequences are included below. Each 2.5ul aliquot of nucleic acid extraction was run with 22.5ul of master mix in triplicate. PCR was run for 32 cycles, initially at 98ºC for 2minutes, with 30 cycles of 98ºC for 30 seconds, 50ºC for 30 seconds, 72ºC for 1 minute and 30 seconds and finished with 72ºC for 5mins. Triplicate PCR products were pooled into single reactions per sample and sample pools were purified using an AMPure XP (Beckman Coulter) workflow at a ratio of 1X. Libraries were quantified using the Qubit High Sensitivity dsDNA kit (ThermoFisher) and equimolar library pools created. The equimolar pools were purified by gel electrophoresis and the Wizard SV Gel and PCR Clean Up Kit (Promega) before submission for sequencing.

Process used in Operations to generate the data

PCR was performed to amplify bacterial 16S ribosomal gene regions using V1V2 specific primers with attached adaptors and indexes. Manually prepared PCR reaction mastermixes were made using the Q5 High-Fidelity Polymerase Kit (New England Biolabs), according to the original protocol as described above. Pre-mixed mastermixes contained 12.5ul Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs) and 7.5ul nuclease free water (ThermoFisher Scientific). 2.5ul nucleic acid extract was used per 25ul triplicate reaction, 4ul per duplicate 40ul reaction and 7.5ul per single 75ul reaction. 1.25ul of each forward and reverse primers (ThermoFisher Scientific) diluted to 10uM with nuclease free water were used per 25ul reaction and scaled accordingly for 40ul and 75ul reactions. Mastermix reagent volumes were scaled accordingly. PCR was run for 30 cycles, initially at 98ºC for

2minutes, with 30 cycles of 98ºC for 30 seconds, 50ºC for 30 seconds, 72ºC for 1 minute and 30 seconds and finished with 72ºC for 5mins. Triplicate and duplicate PCR products were pooled into single reactions per sample respectively and all samples were purified using an AMPure XP (Beckman Coulter) workflow at a ratio of 0.8X. Libraries were quantified using the AccuClear Ultra High Sensitivity dsDNA Quantitation kit (Biotium) and equimolar pools were subsequently created using a Biomek NX-8 liquid handler (Beckman Coulter). Samples were sequenced using the Illumina MiSeq (300bp paired-end reads, v3 Reagent Kit). Process controls included an extraction control, a negative PCR water control, an aliquot of the glycerol used for storage and an aliquot of the water used to dilute the mock community.

Proposed optimisations

Based on our findings, the optimised process would utilise single PCR reactions (75ul), removing the need to set up multiple reactions per sample, significantly saving time and effort. Additionally, this eliminates the requirement for subsequent pooling per sample post-PCR, removing the risk of pooling incorrect samples together, together with the increased level of sample tracking that would otherwise be necessary to support this step at scale. A pre-mixed mastermix would be used in place of a manually prepared mastermix, for speed, convenience and to reduce the risk of manual handling error.

313 **Full length Illumina tagged primers used in study**:

Name	Sequence
V1FW_SD501	AATGATACGGCGACCACCGAGATCTACACAAGCAGCAacactctttcccta cacgacgctcttccgatctNNNNAGMGTTYGATYMTGGCTCAG
V1FW_SD502	AATGATACGGCGACCACCGAGATCTACACACGCGTGAacactctttcccta cacgacgctcttccgatctNNNNAGMGTTYGATYMTGGCTCAG
V1FW_SD503	AATGATACGGCGACCACCGAGATCTACACCGATCTACacactctttcccta cacgacgctcttccgatctNNNNAGMGTTYGATYMTGGCTCAG
V1FW_SD504	AATGATACGGCGACCACCGAGATCTACACTGCGTCACacactctttcccta cacgacgctcttccgatctNNNNAGMGTTYGATYMTGGCTCAG
V1FW_SD505	AATGATACGGCGACCACCGAGATCTACACGTCTAGTGacactctttcccta cacgacgctcttccgatctNNNNAGMGTTYGATYMTGGCTCAG
V1FW_SD506	AATGATACGGCGACCACCGAGATCTACACCTAGTATGacactctttcccta cacgacgctcttccgatctNNNNAGMGTTYGATYMTGGCTCAG
V1FW_SD507	AATGATACGGCGACCACCGAGATCTACACGATAGCGTacactctttcccta cacgacgctcttccgatctNNNNAGMGTTYGATYMTGGCTCAG
V1FW_SD508	AATGATACGGCGACCACCGAGATCTACACTCTACACTacactctttccctac acgacgctcttccgatctNNNNAGMGTTYGATYMTGGCTCAG
V2RV_SD701	CAAGCAGAAGACGGCATACGAGATACCTAGTAgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD702	CAAGCAGAAGACGGCATACGAGATACGTACGTgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD703	CAAGCAGAAGACGGCATACGAGATATATCGCGgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD704	CAAGCAGAAGACGGCATACGAGATCACGATAGgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD705	CAAGCAGAAGACGGCATACGAGATCGTATCGCgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD706	CAAGCAGAAGACGGCATACGAGATCTGCGACTgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD707	CAAGCAGAAGACGGCATACGAGATGCTGTAACgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD708	CAAGCAGAAGACGGCATACGAGATGGACGTTAgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD709	CAAGCAGAAGACGGCATACGAGATGGTCGTAGgtgactggagttcagacgt gtgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD710	CAAGCAGAAGACGGCATACGAGATTAAGTCTCgtgactggagttcagacgtgt gctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD711	CAAGCAGAAGACGGCATACGAGATTACACAGTgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
V2RV_SD712	CAAGCAGAAGACGGCATACGAGATTTGACGCAgtgactggagttcagacgtg tgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT

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Mis-assigned taxa by arb

A few operational taxonomic units were evaluated with BLAST where the species was disconcordant with the taxa genus and/or not a prokaryote, as assigned by arb, or where an uncultured bacterium was seen across the mock microbial community suggesting it was either an expected mock microbial community member or a significant contaminant:

- Experiement 1
    - *Malaclemys terrapin terrapin – E. coli*, OTU 3805
    - uncultured *Enterobacteriaceae bacterium – E. coli* OTU 4256
    - uncultured *Enterobacteriaceae bacterium – E. coli* OTU 4397
    - *Coregonus lavaretus* (common whitefish): *Cutibacterium acnes* OTU 996
    - *Coregonus lavaretus* (common whitefish): uncultured *bacterium* OTU 1020
    - *Coregonus lavaretus* (common whitefish): *Cutibacterium acnes* OTU 4188
    - *Coregonus lavaretus* (common whitefish): uncultured *Propionibacterium* OTU 4327
    - *Coregonus lavaretus* (common whitefish): uncultured *Propionibacterium* OTU 2493
    - *Coregonus lavaretus* (common whitefish): uncultured *Propionibacterium* OTU 4224
    - *Coregonus lavaretus* (common whitefish): uncultured bacterium OTU 2490
    - *Amia calva* (bowfin): *Paracoccus salipaludis* OTU 2864
    - *Ambystoma mexicanum* (axolotl): *Acinetobacter lwoffii* OTU 3596
    - *Ambystoma mexicanum*: *Acinetobacter seifertii* OTU 4146
    - *Ambystoma mexicanum* (axolotl): *Acinetobacter lwoffii* OTU 3597
    - *Solanum melongena* (eggplant): *Moraxella osloensis* OTU 1489
    - unidentified marine bacterioplankton: uncultured *Enterococcaceae* bacterium OTU 4144
    - *Trichuris trichiura* (human whipworm): uncultured *Bacteroidetes* bacterium OTU 1936

- *Trichuris trichiura* (human whipworm): uncultured *Bacteroidetes* bacterium OTU 2223
- *Triticum aestivum* (bread wheat): *Pseudomonas gessardii* OTU 3978
- *Triticum aestivum (*bread wheat): *Pantoea agglomerans* OTU 4362
- *Triticum aestivum* (bread wheat): *Pantoea agglomerans* OTU 4241
- *Athetis lepigone*: *Pseudomonas putida* OTU 2630
- *Elodea nuttallii*: Uncultured *Methyloversatilis* sp. OTU 3029
- *Bryum argenteum var. argenteum*: *Pseudomonas putida* OTU 3620

- Experiment 2:
  - *Malaclemys terrapin terrapin – E. coli*, OTU 1719 - done
  - uncultured *Enterobacteriaceae bacterium – E. coli* OTU 1717
  - *Coregonus lavaretus* (common whitefish): *Cutibacterium acnes* OTU 44 - done
  - *Coregonus lavaretus* (common whitefish): uncultured *bacterium* OTU 1685 - done
  - *Coregonus lavaretus* (common whitefish): uncultured *bacterium* OTU 588 - done
  - *Coregonus lavaretus* (common whitefish): uncultured *Propionibacterium* OTU 1664 - done
  - *Coregonus lavaretus* (common whitefish): uncultured *Propionibacterium* OTU 1820 - done
  - *Coregonus lavaretus* (common whitefish): uncultured *Propionibacterium* OTU 1729 - done
  - *Coregonus lavaretus* (common whitefish): uncultured *Propionibacterium* OTU 591 - done
  - *Coregonus lavaretus* (common whitefish): *Cutibacterium acnes* OTU 1687 - done
  - *Coregonus lavaretus* (common whitefish): uncultured *bacterium* OTU 1751 -

done

- *Solanum melongena* (eggplant): *Moraxella osloensis* OTU 1671
- *Lepisosteus oculatus* (spotted gar): uncultured *Corynebacterium sp.* OTU 1125
- *Lepisosteus oculatus* (spotted gar): *Cutibacterium granulosum* OTU 43
- *Lepisosteus oculatus* (spotted gar): *Cutibacterium granulosum* OTU 1740
- *Lepisosteus oculatus* (spotted gar): uncultured *Corynebacterium sp.* OTU 1128
- *Lepisosteus oculatus* (spotted gar): uncultured *Corynebacterium sp.* OTU 1696
- *Amia calva* (bowfin): *Paracoccus salipaludis* OTU 962
- *Athetis lepigone*: *Pseudomonas putida* OTU 1697
- *Bryum argenteum var. argenteum*: *Pseudomonas putida* OTU 1718 - done
- unidentified marine bacterioplankton: uncultured *Streptococcus sp*. OTU 309

Results from replication study (Experiment 2)

PCR Pooling

After quality filtering of samples used to assess the requirement for pooling of PCR (**Figure 1**), median read counts were 126552, 110890, and 128873 from PCR reactions in triplicate, duplicate or as a single reaction, respectively from experiment 2. Pairwise Mann-Whitney-U test comparisons showed no significant difference in high-quality read counts generated from reactions in triplicate vs duplicate ($p = 0.31$), triplicate vs single ($p=0.42$), or single vs duplicate ($p=0.15$). We then investigated variation in alpha diversity (measures of the within sample diversity). We did not observe any significant difference between PCR pooling strategies using Kruskall-Wallis tests by Shannon, Simpson, Fisher, Chao1, and Observed indices, and replicates from pair-wise PCR pool conditions showed a strong correlation by Kendall's rank correlation coefficient (**Supplementary Table 1, Supplementary Figure 6**). Beta diversity (measure of the similarity or dissimilarity between two samples) by Bray-Curtis

index clustered by replicate on examination of the PCoA and NMDS ordination plots, and did not significantly differ between PCR pooling strategies by PERMANOVA analysis ($F(2)= 0.47$, $p = 0.94$). The groups did differ by PERMANOVA analysis when compared by sample type i.e. mock vs healthy nasal sample ($F(2)= 35.2$, p = <0.001) (**Supplementary Figure 9**). Further, relative abundance of samples by all technical replicates (including various types of mastermix used) appeared to remain similar (**Figure 4 and Figure 5**).

Mastermix preparation

After quality filtering of samples used to assess mastermixes for Experiment 2 (**Figure 1**), difference in read counts from samples with manually prepared mastermix (median = 125222) or premixed mastermix (median = 120012) by Mann-Whitney-U test comparison did not reach statistical significance (p=0.91). Alpha diversity of replicates from manually prepared or premixed mastermix methods by Shannon, Simpson, Fisher, Chao1, and Observed indices, did not significantly differ between by Mann-Whitney-U comparison (**Supplementary Table 3)**. Beta diversity by Bray-Curtis index clustered by mastermix preparation replicate on examination of the PCoA and NMDS ordination plots **(Supplementary Figure 11)**. Further, relative abundance of samples by all technical replicates (including various types of mastermix used) appeared to remain similar **(Figure 3)**. Replicate numbers in the repeat experiment (Experiment 2) were low and examined with the mock community serially diluted samples alone.

CARRIAGE Study Team (in alphabetical order)

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ENA sample accession	Sample site	Experiment
ERS15972987	CARRIAGE	Experiment_1
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ERS15972932	CARRIAGE	Experiment_1
ERS15972931	CARRIAGE	Experiment_1
ERS15972930	CARRIAGE	Experiment_1
ERS15972933	CARRIAGE	Experiment_1
ERS15972934	CARRIAGE	Experiment_1
ERS15972937	CARRIAGE	Experiment_1
ERS15972935	CARRIAGE	Experiment_1
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ERS15972936	CARRIAGE	Experiment_1
ERS15972940	CARRIAGE	Experiment_1
ERS15972939	CARRIAGE	Experiment_1
ERS15972941	CARRIAGE	Experiment_1
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ERS15973092	Mock_Community	Experiment_2
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ERS15973106	Mock_Community	Experiment_2
ERS15973108	Mock_Community	Experiment_2
ERS15973109	Control	Experiment_2

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The STORMS checklist. An editable version for adaptation and inclusion in publications is available from https://stormsmicrobiome.org

Number	Item	Recommendation	Item Source	Additional Guidance	Yes/No/NA	Comments or location in manuscript
Abstract						
1.0	Structured or Unstructured Abstract	Abstract should include information on background, methods, results, and conclusions in structured or unstructured format.	STORMS		Yes	(in accordance to journal requirements)
1.1	Study Design	State study design in abstract.	STORMS	See 3.0 for additional information on study design.	NA	Not required by journal
1.2	Sequencing methods	State the strategy used for metagenomic classification.	STORMS	For example, targeted 16S by qPCR or sequencing, shotgun metagenomics, metatranscriptomics, etc.	Yes	Abstract
1.3	Specimens	Describe body site(s) studied.	STORMS		Yes	Abstract
Introduction						
2.0	Background and Rationale	Summarize the underlying background, scientific evidence, or theory driving the current hypothesis as well as the study objectives.	STORMS		Yes	Background
2.1	Hypotheses	State the pre-specified hypothesis. If the study is exploratory, state any pre-specified study objectives.	STORMS		Yes	Background
Methods						
3.0	Study Design	Describe the study design.	STORMS	Observational (Case-Control, Cohort, Cross-sectional survey, etc.) or Experimental (Randomized controlled trial, Non-randomized controlled trial, etc.). For a brief description of common study designs see: DOI: 10.11613/BM.2014.022 If applicable, describe any blinding (e.g. single or double-blinding) used in the course of the study.	Yes	Methods

3.1	Participants	State what the population of interest is, and the method by which participants are sampled from that population. Include relevant information on physiological state of the subjects or stage in the life history of disease under study when participants were sampled.	STORMS	Examples of the population of interest could be: adults with no chronic health conditions, adults with type II diabetes, newborns, etc. This is the total population to whom the study is hoped to be generalizable to. The sampling method describes how potential participants were selected from that population. If the participants are from a substudy of a larger study, provide a brief description of that study and cite that study. Clearly state how cases and controls are defined. An example of relevant physiological state might be pre/post menopausal for a vaginal microbiome study; examples of stage in the life history of disease could be whether specimens were collected during active or dormant disease, or before or after treatment.	Yes	Methods
3.2	Geographic location	State the geographic region(s) where participants were sampled from.	MIxS: geographic location (country and/or sea,region)	Geographic coordinates can be reported to prevent potential ambiguities if necessary.	Yes	Methods
3.3	Relevant Dates	State the start and end dates for recruitment, follow-up, and data collection.	STORMS	Recruitment is the period in which participants are recruited for the study. In longitudinal studies, follow-up is the date range in which participants are asked to complete a specific assessment. Finally, data collection is the total period in which data is being collected from participants including during initial recruitment through all follow-ups.	Yes	Methods

3.4	Eligibility criteria	List any criteria for inclusion and exclusion of recruited participants.	Modified STROBE	Among potential recruited participants, how were some chosen and others not? This could include criteria such as sex, diet, age, health status, or BMI. If there is a primary and validation sample, describe inclusion/exclusion criteria for each.	NA	
3.5	Antibiotics Usage	List what is known about antibiotics usage before or during sample collection.	STORMS	If participants were excluded due to current or recent antibiotics usage, state this here. Other factors (e.g. proton pump inhibitors, probiotics, etc.) that may influence the microbiome should also be described as well.	NA	
3.6	Analytic sample size	Explain how the final analytic sample size was calculated, including the number of cases and controls if relevant, and reasons for dropout at each stage of the study. This should include the number of individuals in whom microbiome sequencing was attempted and the number in whom microbiome sequencing was successful.	STORMS	Consider use of a flow diagram (see template at https://stormsmicrobiome.org/figures). Also state sample size in abstract. If power analysis was used to calculate sample size, describe those calculations.	Yes	Methods
3.7	Longitudinal Studies	For longitudinal studies, state how many follow-ups were conducted, describe sample size at follow-up by group or condition, and discuss any loss to follow-up.	STORMS	If there is loss to follow-up, discuss the likelihood that drop-out is associated with exposures, treatments, or outcomes of interest.	NA	NA
3.8	Matching	For matched studies, give matching criteria.	Modified STROBE	"Matched" refers to matching between comparable study participants as cases and controls or exposed / unexposed. Indicate whether participants were individual or frequency matched and in what ratio were they matched (e.g. 1 case to 1 control).	NA	NA
3.9	Ethics	State the name of the institutional review board that approved the study and protocols, protocol number and date of approval, and procedures for obtaining informed consent from participants.	STORMS		Yes	Methods and Declarations
4.0	Laboratory methods	State the laboratory/center where laboratory work was done.	STORMS	Provide a reference to complete lab protocols if previously published elsewhere such as on protocols.io. Note any modifications of lab protocols	Yes	Methods

				and the reason for protocol modifications.		
4.1	Specimen collection	State the body site(s) sampled from and how specimens were collected.	MIxS: sample collection device or method; host body site	Use terms from the Uber-anatomy Ontology (https://www.ebi.ac.uk/ols/ontologies/uberon) to describe body sites in a standardized format.	Yes	Methods
4.2	Shipping	Describe how samples were stored and shipped to the laboratory.	STORMS	Include length of time from collection to receipt by the lab and if temperature control was used during shipping.	Yes	Methods
4.3	Storage	Describe how the laboratory stored samples, including time between collection and storage and any preservation buffers or refrigeration used.	STORMS	State where each procedure or lot of samples was done if not all in the same place. Include reagent/lot/catalogue #s for storage buffers.	Yes	Methods
4.4	DNA extraction	Provide DNA extraction method, including kit and version if relevant.	MIxS: nucleic acid extraction	If any DNA quantification methods were used prior to DNA amplification or at the pooling step of library preparation, state so here.	Yes	Methods
4.5	Human DNA sequence depletion or microbial DNA enrichment	Describe whether human DNA sequence depletion or enrichment of microbial or viral DNA was performed.	STORMS		NA	NA
4.6	Primer selection	Provide primer selection and DNA amplification methods as well as variable region sequenced (if applicable).	MIxS: pcr primers		Yes	Methods
4.7	Positive Controls	Describe any positive controls (mock communities) if used.	STORMS	If used, should be deposited under guidance provided in the 8.X items.	Yes	Methods
4.8	Negative Controls	Describe any negative controls if used.	STORMS	If used, should be deposited under guidance provided in the 8.X items.	Yes	Methods
4.9	Contaminant mitigation and identification	Provide any laboratory or computational methods used to control for or identify microbiome contamination from the environment, reagents, or laboratory.	STORMS	Includes filtering of reagents and other steps to minimize contamination. It is relevant to state whether the specimens of interest have low microbial load, which makes contamination especially relevant.	Yes	Methods and Results

4.10	Replication	Describe any biological or technical replicates included in the sequencing, including which steps were replicated between them.	STORMS	Replication may be biological (redundant biological specimens) or technical (aliquots taken at different stages of analysis) and used in extraction, sequencing, preprocessing, and/or data analysis.	Yes	Methods and Results
4.11	Sequencing strategy	Major divisions of strategy, such as shotgun or amplicon sequencing.	MIxS: sequencing method	For amplicon sequencing (for example, 16S variable region), state the region selected. State the model of sequencer used.	Yes	Methods
4.12	Sequencing methods	State whether experimental quantification was used (QMP/cell count based, spike-in based) or whether relative abundance methods were applied.	STORMS	These include read length, sequencing depth per sample (average and minimum), whether reads are paired, and other parameters.	Yes	Methods
4.13	Batch effects	Detail any blocking or randomization used in study design to avoid confounding of batches with exposures or outcomes. Discuss any likely sources of batch effects, if known.	STORMS	Sources of batch effects include sample collection, storage, library preparation, and sequencing and are commonly unavoidable in all but the smallest of studies.	Yes	Results
4.14	Metatranscriptomics	Detail whether any mRNA enrichment was performed and whether/how retrotranscription was performed prior to sequencing. Provide size range of isolated transcripts. Describe whether the sequencing library was stranded or not. Provide details on sequencing methods and platforms.	STORMS	Provide details on any internal standards which may have been used as well as parameters and versions of any software or databases used.	NA	NA
4.15	Metaproteomics	Detail which protease was used for digestion. Provide details on proteomic methods and platforms (e.g. LC-MS/MS, instrument type, column type, mass range, resolution, scan speed, maximum injection time, isolation window, normalised collision energy, and resolution).	STORMS	Provide details on any internal standards which may have been used as well as parameters and versions of any software or databases used.	NA	NA
4.16	Metabolomics	Specify the analytic method used (such as nuclear magnetic resonance spectroscopy or mass spectrometry). For mass spectrometry, detail which fractions were obtained (polar and/or non-polar) and how these were analyzed. Provide details on metabolomics methods and platforms (e.g. derivatization, instrument type, injection type, column type and instrument settings).	STORMS	Provide details on any internal standards which may have been used as well as parameters and versions of any software or databases used.	NA	NA

5.0	Data sources/ measurement	For each non-microbiome variable, including the health condition, intervention, or other variable of interest, state how it was defined, how it was measured or collected, and any transformations applied to the variable prior to analysis.	MIxS: host disease status	State any sources of potential bias in measurements, for example multiple interviewers or measurement instruments, and whether these potential biases were assessed or accounted for in study design. Use terms from a standardized ontology such as the Experimental Factor Ontology (https://www.ebi.ac.uk/efo/) to describe variables of interest in a standardized format.	Yes	Methods
6.0	Research design for causal inference	Discuss any potential for confounding by variables that may influence both the outcome and exposure of interest. State any variables controlled for and the rationale for controlling for them.	STORMS	For causal inference, this item refers to describing the assumptions that would be required to draw causal inferences from observational data. See Vujkovic-Cvijin, I., Sklar, J., Jiang, L. et al. Host variables confound gut microbiota studies of human disease. Nature 587, 448–454 (2020). https://doi.org/10.1038/s41586-020-2881-9 for more details on confounding in observational microbiome studies. For example, hypothesized confounders may be controlled for by multivariable adjustment. Consider using a directed acyclic graph (DAG) to describe your causal model and justify any variables controlled for. DAGs can be made using www.dagitty.net.	NA	NA
6.1	Selection bias	Discuss potential for selection or survival bias.	STORMS	Selection bias can occur when some members of the target study population are more likely to be included in the study/final analytic sample than others. Some examples include survival bias (where part of the target study population is more likely to die before they can be studied), convenience sampling (where members of the target study population are not selected at random), and loss to follow-up (when probability of dropping out is related to one of the things being studied).	NA	NA

7.0	Bioinformatic and Statistical Methods	Describe any transformations to quantitative variables used in analyses (e.g. use of percentages instead of counts, normalization, rarefaction, categorization).	STORMS	If a variable is analyzed using different transformations, state rationale for the transformation and for each analyses which version of the variable is used. In case of any complex or multistep transformations, give enumerated instructions for reproducing those transformations.	Yes	Methods and Results
7.1	Quality Control	Describe any methods to identify or filter low quality reads or samples.	MIxS: sequence quality check	If samples were excluded based on quality or read depth, list the criteria used, the number of samples excluded, and the final sample size after quality control.	Yes	Methods
7.2	Sequence analysis	Describe any taxonomic, functional profiling, or other sequence analysis performed.	MIxS: feature prediction; similarity search method		Yes	Methods
7.3	Statistical methods	Describe all statistical methods.	Modified STROBE	Describe any statistical tests used, exploratory data analysis performed, dimension reduction methods/unsupervised analysis, alpha/beta metrics, and/or methods for adjusting for measurement bias. If multiple statistical methods are possible, discuss why the methods used were selected. If a multiple hypothesis testing correction method was used, describe the type of correction used. State which taxonomic levels are analyzed.	Yes	Methods
7.4	Longitudinal analysis	If the study is longitudinal, include a section that explicitly states what analysis methods were used (if any) to account for grouping of measurements by individual or patterns over time.	STORMS		NA	NA
7.5	Subgroup analysis	Describe any methods used to examine subgroups and interactions.	STROBE		NA	NA

7.6	Missing data	Explain how missing data were addressed.	STROBE	"Missing data" refers to participant measurements such as covariates, exposures, outcomes, or time points that should have been collected but were not, not to zeros in taxonomic abundance tables or data points not applicable to that observation.	NA	NA
7.7	Sensitivity analyses	Describe any sensitivity analyses.	STROBE		Yes	Methods (mock microbial community in dilutions)
7.8	Findings	State criteria used to select findings for reporting.	STORMS	For example, false discovery rate with total number of tests, effect size threshold, significance threshold, microbes of interest.	Yes	Methods
7.9	Software	Cite all software (including read mapping software) and databases (including any used for taxonomic reference or annotating amplicons, if applicable) used. Include version numbers.	Modified STREGA	Installed packages, add-ons or libraries should be stated and cited in addition to the software used. All parameters employed that differ from the default of that software/version should be provided. This is in addition to, not a replacement for, publishing of code as outlined in the section Reproducible Research.	Yes	Methods
8.0	Reproducible research	Make a statement about whether and how others can reproduce the reported analysis.	STORMS	Any protected information that has been excluded or provided under controlled access should be listed along with any relevant data access procedures. "On request from authors" is not sufficiently detailed; formal data access procedures and conditions should be defined. If data are unavailable, state so clearly. Consider using a specialized rubric for reproducible research (such as: https://mbio.asm.org/content/9/3/e00525-18.short).	Yes	Data Availability

				Consider preregistering the study protocol (such as on osf.io or https://plos.org/open-science/preregistration/).		
8.1	Raw data access	State where raw data may be accessed including demultiplexing information.	STORMS	Robust, long-term databases such as those hosted by NCBI and EBI are preferred. If using a private repository, provide rationale.	Yes	Data Availability
8.2	Processed data access	State where processed data may be accessed.	STORMS	Unfiltered data should be provided. Robust, long-term databases such as those hosted by NCBI and EBI-EMBL are preferred. Repositories like zenodo (https://zenodo.org/) or publisso (https://www.publisso.de/en/working-for-you/doi-service/) can be used to provide a DOI and long-term storage for processed datasets, even those which cannot be published openly.	NA	NA
8.3	Participant data access	State where individual participant data such as demographics and other covariates may be accessed, and how they can be matched to the microbiome data.	STORMS	If re-categorized, transformed, or otherwise derived variables were used in the analysis, these variables or code for deriving them should be provided. Examples of how participant data can be matched to microbiome data are: using the same set of anonymized identifiers, or using different anonymized identifiers but providing a map.	NA	NA

				Provided data should be sufficient to independently replicate the current analysis.		
8.4	Source code access	State where code may be accessed.	STORMS	If a standard or formalized workflow was employed, reference it here.	NA	NA
8.5	Full results	Provide full results of all analyses, in computer-readable format, in supplementary materials.	STORMS	For example, any fold-changes, p-values, or FDR values calculated, provided as a spreadsheet. Use a machine-readable, plain-text format such as csv or tsv.	NA	NA
Results						
9.0	Descriptive data	Give characteristics of study participants (e.g. dietary, demographic, clinical, social) and information on exposures and potential confounders.	STROBE	Typically reported in a table included in the paper or as a supplementary table. Indicate number of participants with missing data for each variable of interest. This includes environmental and lifestyle factors that may affect the relationship between the microbiome and the condition of interest. Participant diet and medication use should be summarized, if known. At minimum, age and sex of all participants should be summarized.	Yes	Methods
10.0	Microbiome data	Report descriptive findings for microbiome analyses with all applicable outcomes and covariates.	STORMS	This includes measures of diversity as well as relative abundances. These descriptive findings should be reported both for the sample overall and for individual groups.	Yes	Results

10.1	Taxonomy	Identify taxonomy using standardized taxon classifications that are sufficient to uniquely identify taxa.	STORMS	If not using full taxonomic hierarchy, make sure it is clear whether names stated are species, genera, family, etc. Italicize genus/species pairs. Consult journal guidelines or standardized references on taxonomic nomenclature. For instance, https://wwwnc.cdc.gov/eid/page/scientific-nomenclature	Yes	Results
10.2	Differential abundance	Report results of differential abundance analysis by the variable of interest and (if applicable) by time, clearly indicating the direction of change and total number of taxa tested.	STORMS	If there are more than two groups, include omnibus (multigroup) test results if applicable to the research question. If applicable, reported effect sizes should include a measure of uncertainty such as the confidence interval.	Yes	Results
10.3	Other data types	Report other data analyzed--e.g. metabolic function, functional potential, MAG assembly, and RNAseq.	STORMS		NA	NA
10.4	Other statistical analysis	Report any statistical data analysis not covered above.	STORMS	This could include subgroup analysis, sensitivity analyses, and cluster analysis. Visualizations should be easily interpretable and colorblind-friendly. The caption and/or main text should provide a detailed description of visualizations for visually-impaired readers.	Yes	Results
Discussion						
11.0	Key results	Summarise key results with reference to study objectives	STROBE		Yes	Discussion

12.0	Interpretation	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	STROBE	Define or clarify any subjective terms such as "dominant," "dysbiosis," and similar words used in interpretation of results. When interpreting the findings, consider how the interpretation of the findings may be summarized or quoted for the general public such as in press releases or news articles. If causal language is used in the interpretation (such as "alters," "affects," "results in," "causes," or "impacts"), assumptions made for causal inference should be explicitly stated as part of 6.0 and 13.0. Distinguish between function potential (ie inferred from metagenomics) and observed activity (ie metatranscriptomic, metabolomic, proteomic) if discussing microbial function.	Yes	Discussion
13.0	Limitations	Discuss limitations of the study, taking into account sources of potential bias or imprecision.	STROBE	Also consider limitations resulting from the methods (especially novel methods), the study design, and the sample size.	Yes	Discussion
13.1	Bias	Discuss any potential for bias to influence study findings.	STORMS	May include sampling method, representativeness of study participants, or potential confounding.	Yes	Discussion
13.2	Generalizability	Discuss the generalisability (external validity) of the study results	STROBE	To what populations or other settings do you expect the conclusions to generalize?	Yes	Discussion
14.0	Ongoing/future work	Describe potential future research or ongoing research based on the study's findings.	STORMS		Yes	Discussion
Other information						
15.0	Funding	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	STROBE		Yes	Funding

15.1	Acknowledgements	Include acknowledgements of those who contributed to the research but did not meet critera for authorship.	STORMS	For general guidelines on authorship, see http://www.icmje.org and https://www.elsevier.com/authors/journal-authors/policies-and-ethics/credit-author-statement	Yes	Acknowledgements
15.2	Conflicts of Interest	Include a conflicts of interest statement.	STORMS		Yes	Competing interests
16.0	Supplements	Indicate where supplements may be accessed and what materials they contain.	STORMS		Yes	Supplementary data
17.0	Supplementary data	Provide supplementary data files of results with for all taxa and all outcome variables analyzed. Indicate the taxonomic level of all taxa.	STORMS	Depending on the analysis performed, examples of the supplemental results included could be mean relative abundance, differential abundance, raw p-value, multiple hypothesis testing-adjusted p-values, and standard error. All discussed taxa should include the taxonomic level (e.g. class, order, genus).	Yes	