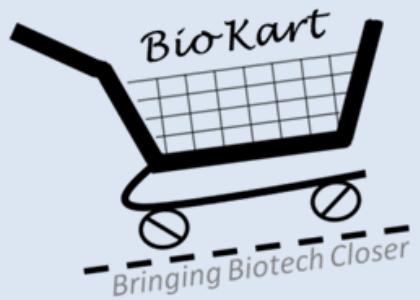


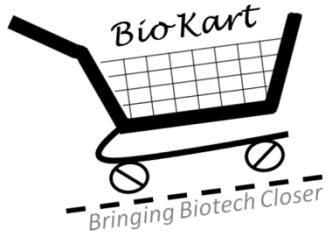


16s Metagenomics Report



Biokart India Pvt. Ltd.

Bringing Biotech Closer



METAGENOMICS _ REPORT

Client Details: Dr. Madhumita Priyadarsini

Total Samples : 2

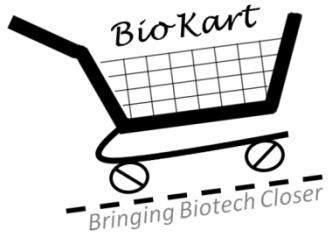
Sample Names: NG-ADS30thDay, NG-CD30thDay

Project ID: BKMTG2588

Date of Report Generation: 21-07-2023

Services Availed:

- DNA Extraction
- DNA QC
- PCR amplification with V3-V4 Primers
- PCR Product Purification
- Sequencing using Illumina MiSeq Platform
- Data Analysis & Report Generation

**Important note:**

Our Standard protocol for report making includes the following:

Projects with 1 sample:

1. QC
2. Taxonomic barplots
3. Top 10 pie chart
4. OTU table

Projects with 2 samples:

1. QC
2. Taxonomic barplots
3. Top 10 pie chart (for each sample)
4. Venn diagram
5. OUT table
6. (Alpha diversity and Rarefaction curve can be provided only upon request)

Projects with 3 samples:

1. QC
2. Taxonomic barplots
3. Top 10 pie chart (for each sample)
4. Venn diagram
5. OUT table
6. (Alpha diversity ,Rarefaction curve ,heatmap, dendrogram and PCA plot can be provided only upon request)



METAGENOMICS _ REPORT

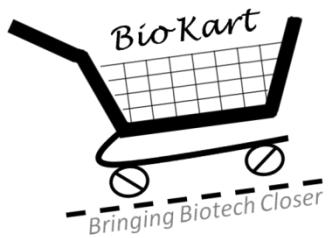
Projects with 4 samples:

When we are having samples count of 4 and above we can consider grouping the samples. If you don't want the samples to be grouped we can provide the following information accordingly,

1. QC
2. Taxonomic barplots
3. Top 10 pie chart (for each sample)
4. Venn diagram
5. OTU table
6. (Alpha diversity ,Rarefaction curve , heatmap, dendrogram, Core microbiome and PCA plot can be provided only upon request)

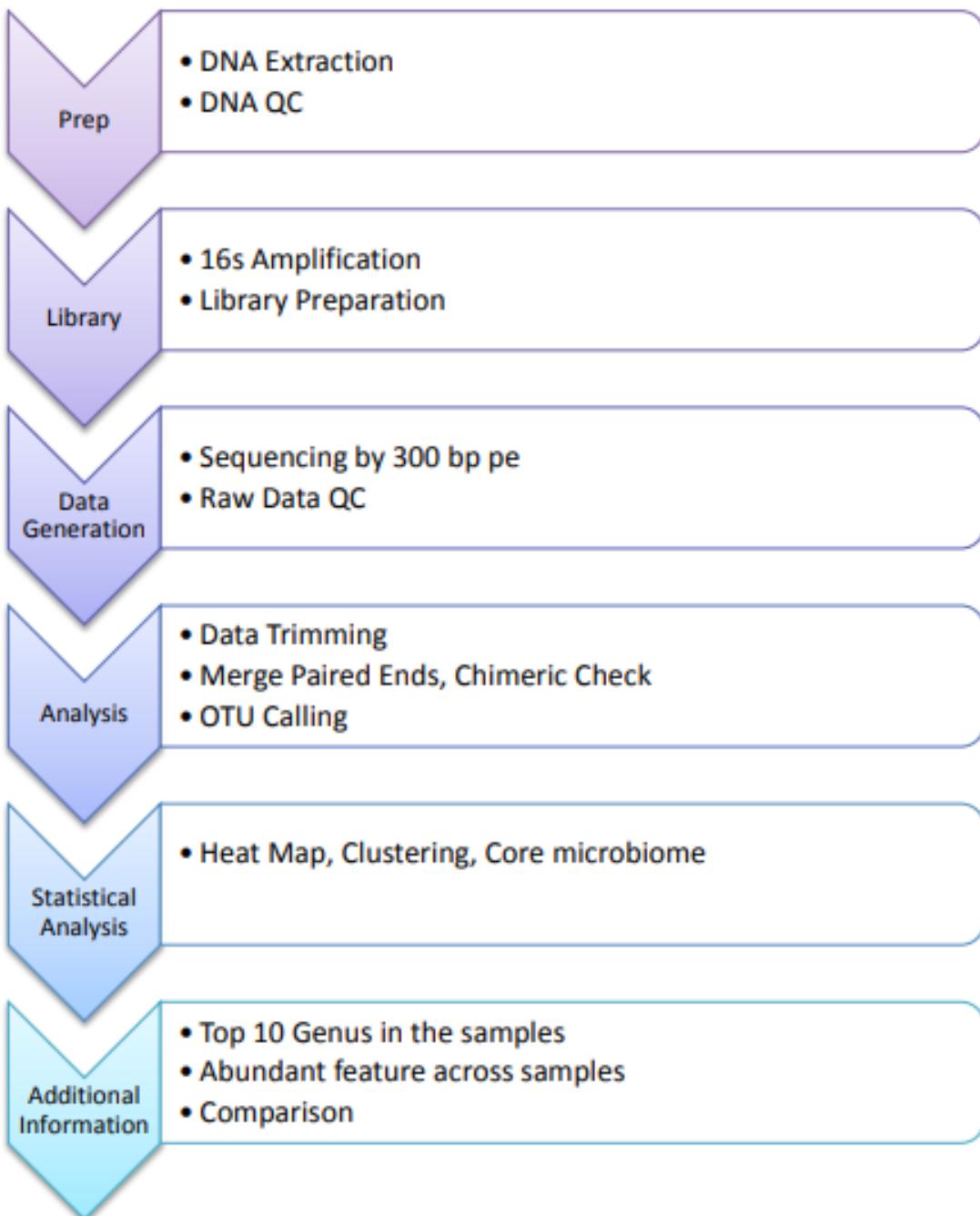
If you want the samples to be analyzed based on certain category or group, we can provide you the following details,

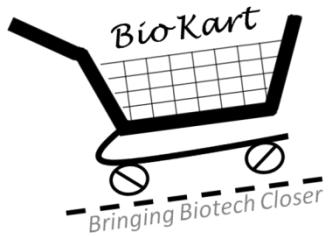
1. QC
2. Taxonomic barplots
3. Top 10 pie chart (for each sample)
4. Venn diagram
5. OTU table
6. Rarefaction curve, Diversity (Alpha and Beta)
7. PCA plot
8. Heat map, Dendrogram, Core microbiome
9. Kegg metabolic pathway
10. Correlation plot
11. LEfSe plot
12. Venn diagram (Will not be provided for projects with more than 4 samples.



METAGENOMICS _ REPORT

Pipeline



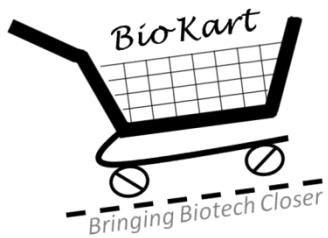


METAGENOMICS _ REPORT

DNA Extraction : (Optional)

DNA extraction is done using Xploregen kit.

- Add 60 ul of solution C1 and invert several times or vortex briefly [Note: Solution C1 maybe added to the power bead tube before adding sample]
 - Vortex at max speed for 10 mins. [Note: If using the 24-place vortex adapter for more than 12 preps, increase the vortex time by 5-10 mins]
 - Centrifuge tube at 10,000 x g for 30s
 - Transfer the supernatant to a clean 2ml collection tube. [Note: expect between 400 to 500 ul of supernatant. Supernatant may still contain some particles.]
 - Add 250 ul of soln. C2 and vortex for 5 sec. Incubate at 4 degree C for 5 mins.
 - Centrifuge the tube for 1 min at 10,000 X g
 - Avoiding the palette, transfer up to 600 ul of supernatant to a clean 2ml collection tube.
 - Add 200 ul of soln. C3 and vortex briefly. Incubate at 4 degree C for 5 mins. [Note: You can skip 5 min incubation. However, if you have already validated the extraction with this incubation, we recommend you to retain the step.]
 - Centrifuge the tube for 1 min at 10,000 X g.
 - Avoiding the palette, transfer up to 600 ul of supernatant to a clean 2ml collection tube.
 - Shake to mix soln. C4 and add 1200ul to the supernatant. Vortex for 5 s.
 - Load 675ul onto an MB Spin Column and centrifuge at 10,000 X g for 1 min. Discard flow through.
 - Repeat the above step twice, until all of the samples have been processed.
 - Add 500 ul of the solution C5. Centrifuge for 30s at 10,000 X g.
 - Discard the flow through. Centrifuge again for 1 min at 10,000 X g.
 - Carefully place the MB spin column into a clean 2ml collection tube. Avoid splashing any solution C5 onto the column.
 - Add 100ul of the soln. C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-free PCR grade water for this step.
 - Centrifuge at room temperature for 30s at 10,000 X g. Discard the MB spin column.
- The DNA is now ready for downstream application. [Note: Solution C6 is 10 mM Tris-HCl, pH 8.5. We recommend storing DNA frozen (-20 degree C - -80 degree C) as soln. C6 does not contain EDTA.]

**Role of buffer:**

- C1 buffer- Lysis of cell
- C2 buffer- removal of Proteins and RNA
- C3 buffer- to remove other residues
- C4 buffer- binding buffer (binds with DNA)
- C5 buffer- wash buffer (to wash DNA)
- C6 buffer- Elution of DNA

PCR Amplification of 16s Region:

Composition of TAQ Master MIX:

- 1) High-Fidelity DNA Polymerase
- 2) 0.5mM dNTPs
- 3) 3.2mM MgCl₂
- 4) PCR Enzyme Buffer

Primer Details:

16sF:- 5' AGAGTTGATGMTGGCTCAG3'

16sR:- 5' TTACCGCGGCMGCSGGCAC3'

Conditions Used:

40ng of Extracted DNA is used for amplification along with 10pM of each primer

Initial Denaturation - 95 degree C

25 Cycles of the following condition,

Denaturation @ 95 deg C for 15 sec, Annealing @ 60 deg C for 15 sec, Elongation @ 72 deg C for 2 mins

Final Extension at 72 deg C for 10 mins and Hold at 4 deg C.



METAGENOMICS _ REPORT

DNA QC :

Extracted DNA from the samples were subjected to Nano Drop and GEL Check before being taken for PCR amplification:

The Nano Drop readings of 260/280 at an ~ value of 1.8 to 2 is used to determine the DNA's quality

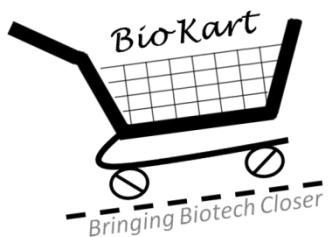
PCR Ampliqon QC:

The amplified 16s PCR Product is purified and subjected to GEL Check and Nanodrop QC

The Nano Drop readings of 260/280 at an ~ value of 1.8 to 2 is used to determine the DNA's quality

Overview of Sequencing Protocol:

The amplicons from each sample were purified with Ampure beads to remove unused primers and an additional 8 cycles of PCR was performed using Illumina barcoded adapters to prepare the sequencing libraries. Libraries were purified using Ampure beads and quantitated using Qubit dsDNA High Sensitivity assay kit. Sequencing was performed using Illumina Miseq with 2x300PE v3-v4 sequencing kit.



Bioinformatics Protocol | Metagrnomics:

The database used for 16s V3-V4 region is NCBI

Step1:

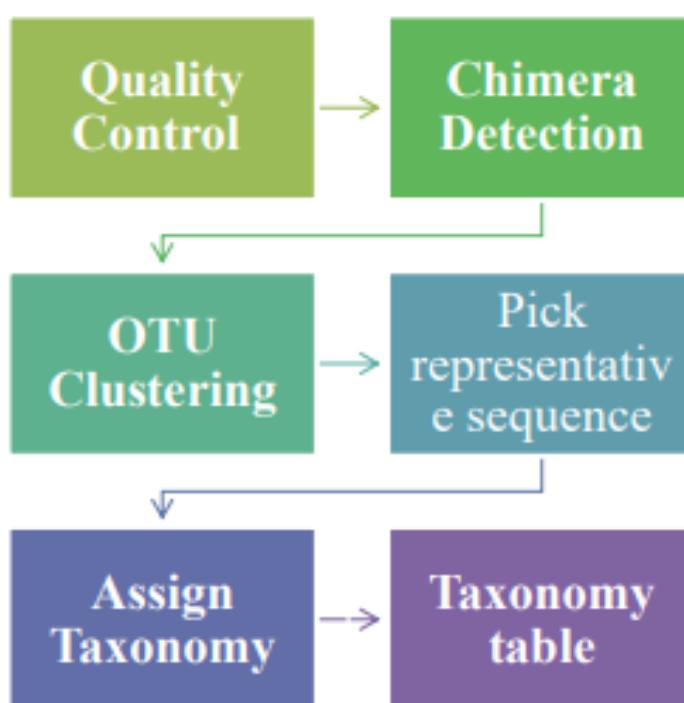
The bcl data received from the sequencer is de-multiplexed into .fastq raw data.

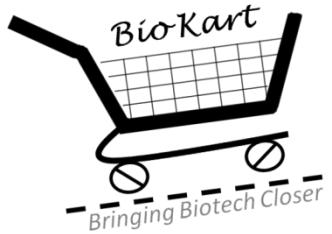
Step2:

The de-multiplexed data quality will be checked using Fastqc (Version 0.11.9) and Multiqc (Version 1.10.1) tools.

Step3:

The QC passed samples are qualified for further analysis and we use our own pipeline for metagenomics (Biokart Pipeline) for 16s metagenomic. The workflow of the pipeline is as follows,





METAGENOMICS _ REPORT

Step4:

Once the run is over we get the final raw OTU table from which we start visualization of the analysis.

Step5:

The abundance feature tables and the top ten organisms in each samples are constructed using Microsoft excel (2016).

Step6:

The other analyses like Heatmap, core microbiome, Dendrogram, Alpha diversity, Beta diversity, PCOA plot, Rarefaction curve, KEGG pathway etc. were built using Microbiome analyst (online tool: <https://www.microbiomeanalyst.ca/>).

***Note:

The report type changes for every project depending on the number of samples provided.



METAGENOMICS _ REPORT

METAGENOMICS REPORTSample Details for V3-V4 amplicon region

Sl. No.	Sample	GC Content (%)	Read Count (in Million)
1	NG-ADS30thDay	53%	0.2
2	NG-CD30thDay	53%	0.4

FastQC (v0.11.2) and MultiQC (version 1.9):

To do quality control checks on raw sequence data coming from high throughput sequencing pipelines.
Multiqc consolidates fastqc results into single report.

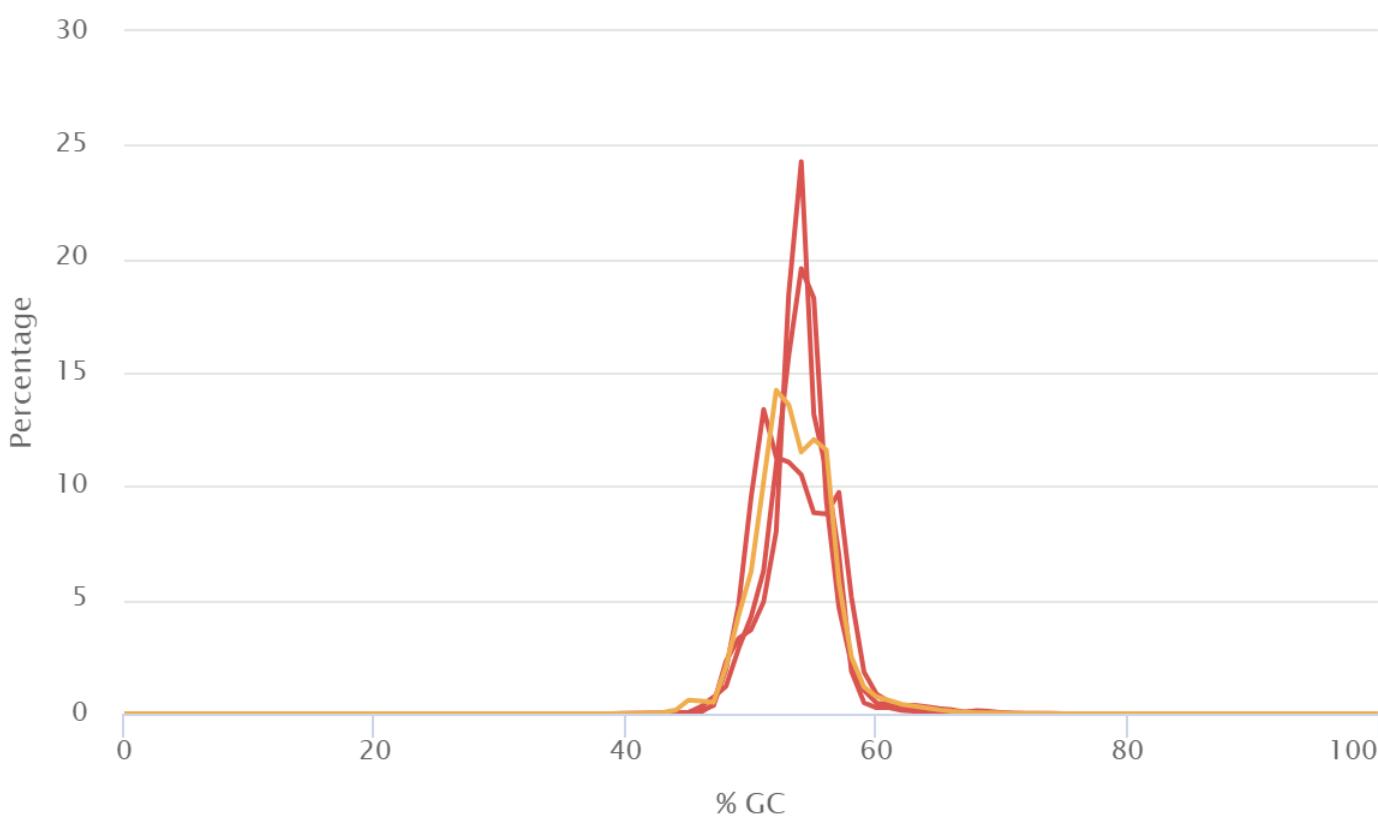




METAGENOMICS _ REPORT

METAGENOMICS REPORT**GC Content**

FastQC: Per Sequence GC Content



Created with MultiQC





Biokart India Pvt Ltd

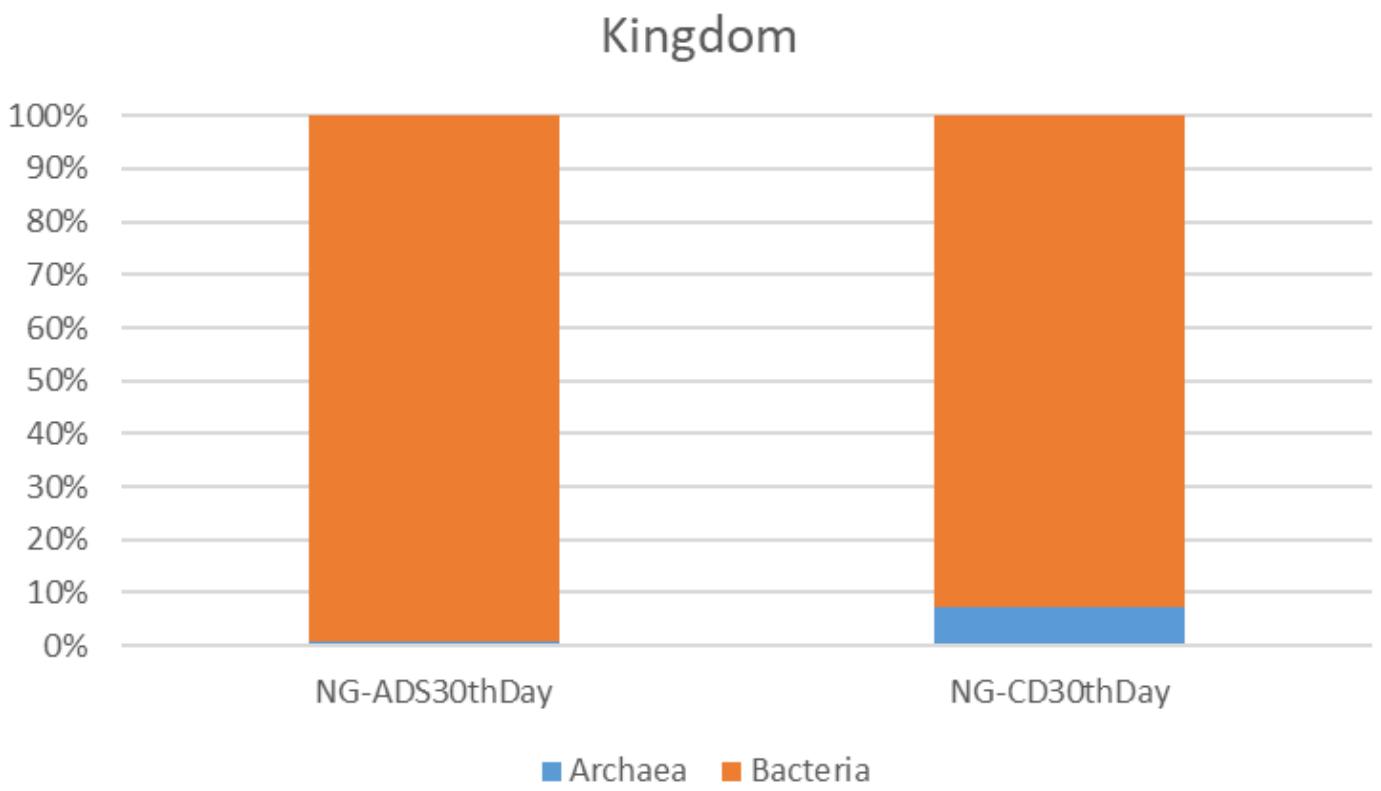
METAGENOMICS _ REPORT

METAGENOMICS REPORT

TAXONOMIC PROFILE



METAGENOMICS _ REPORT

METAGENOMICS REPORTKingdom Level – Taxonomy Plot Analysis



Biokart India Pvt Ltd

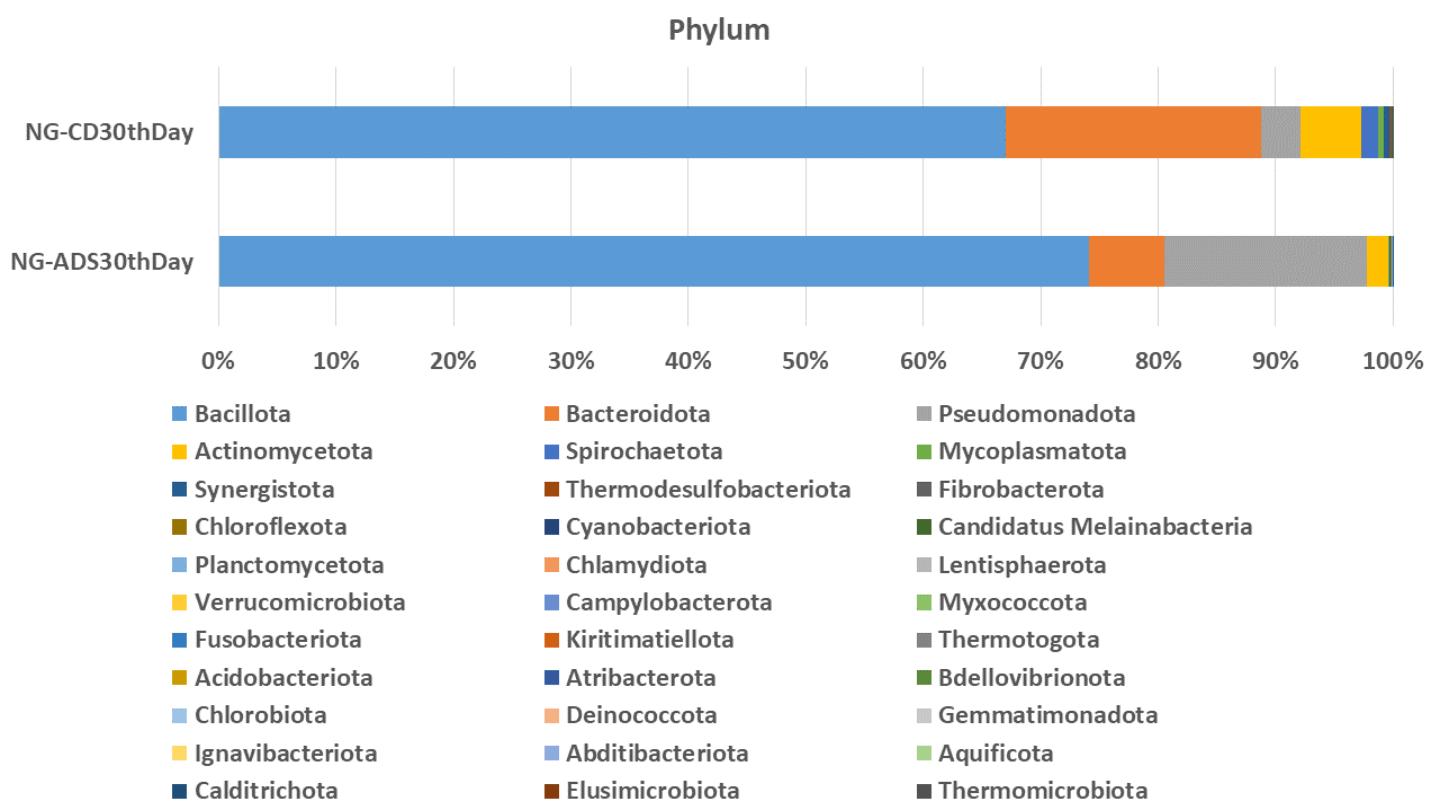
METAGENOMICS _ REPORT

METAGENOMICS REPORT

BACTERIA - TAXONOMIC PROFILE

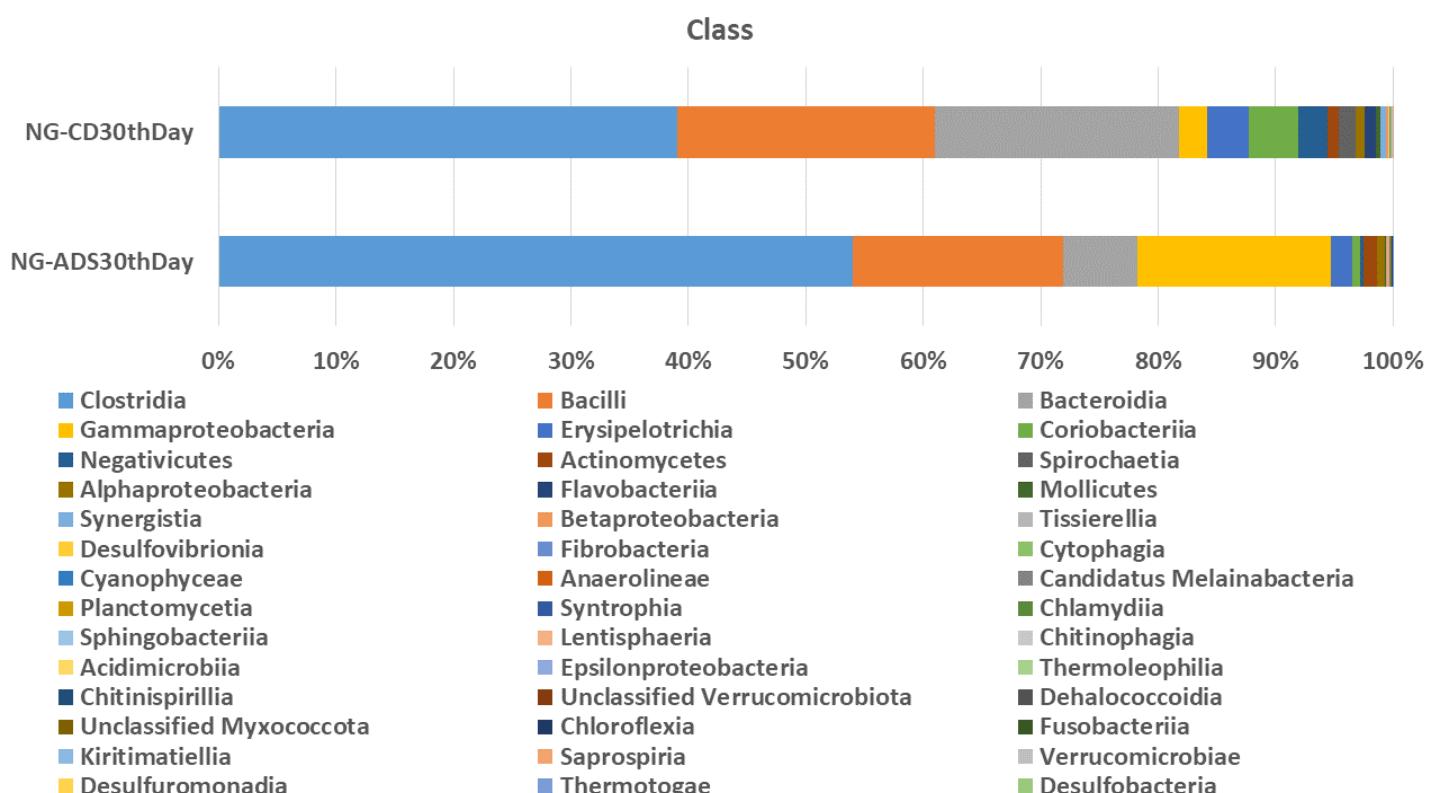


METAGENOMICS _ REPORT

METAGENOMICS REPORTPhylum Level – Taxonomy Plot Analysis

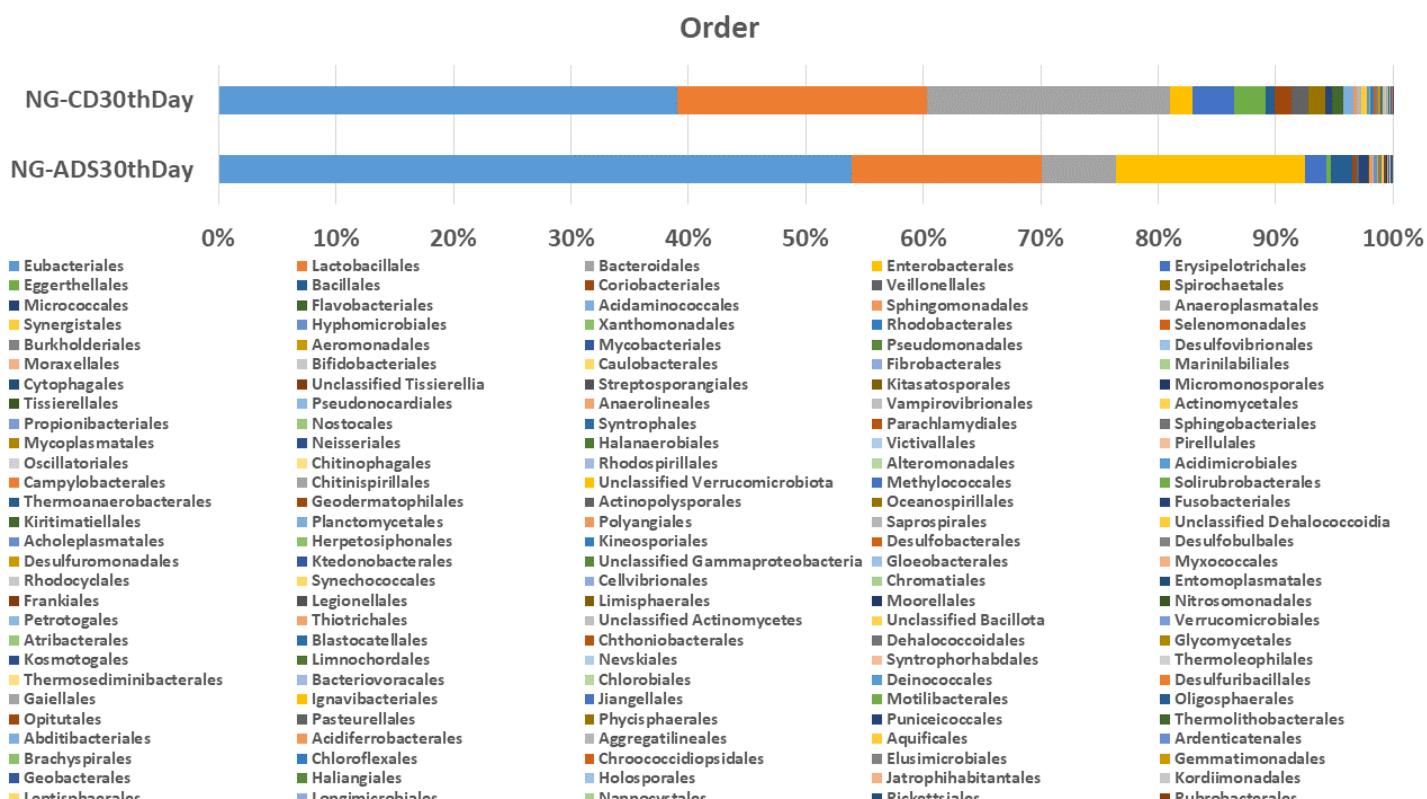


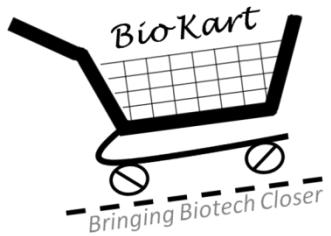
METAGENOMICS _ REPORT

METAGENOMICS REPORTClasses Level – Taxonomy Plot Analysis

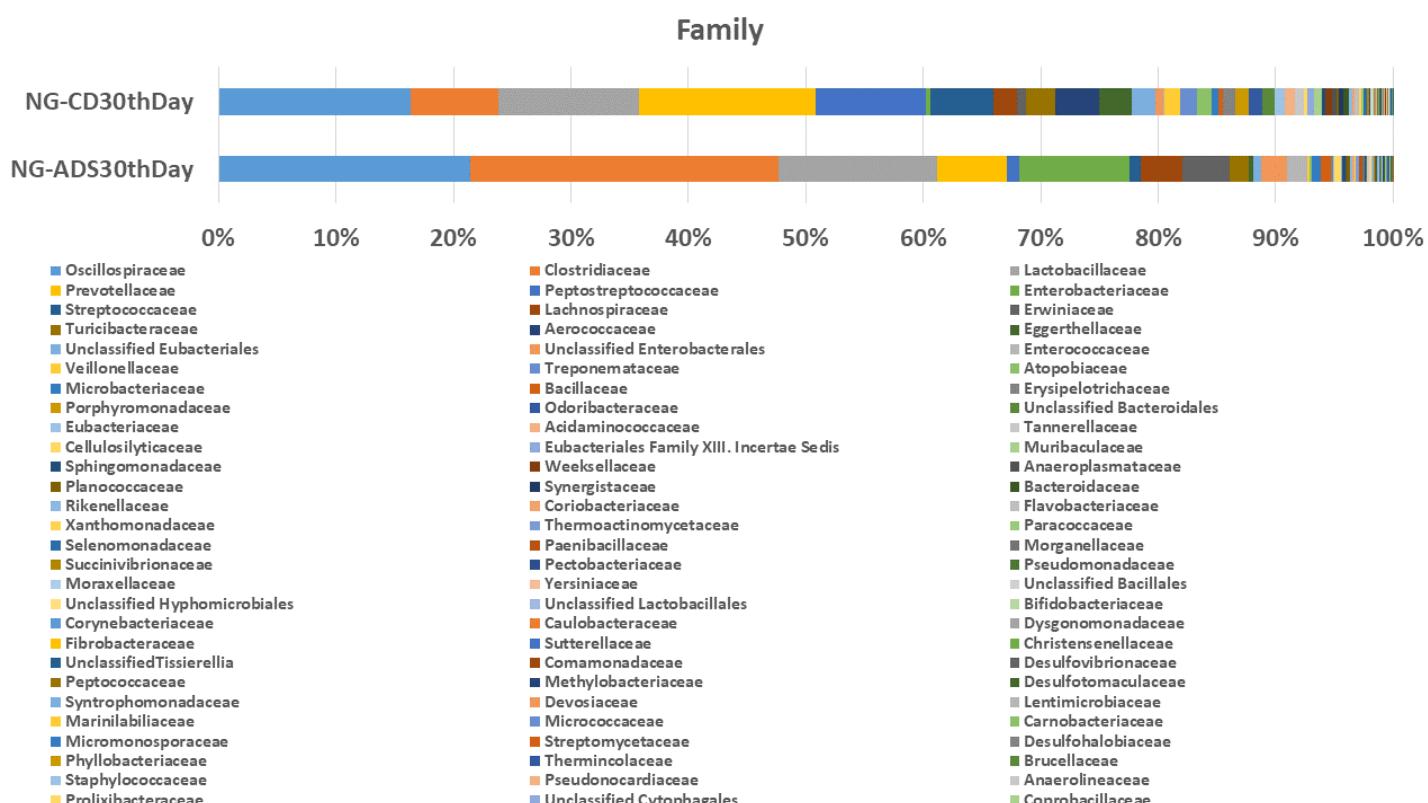


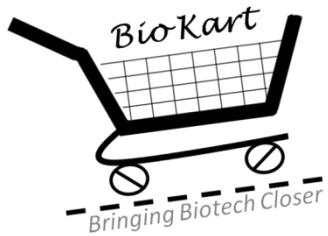
METAGENOMICS _ REPORT

METAGENOMICS REPORTOrder Level – Taxonomy Plot Analysis

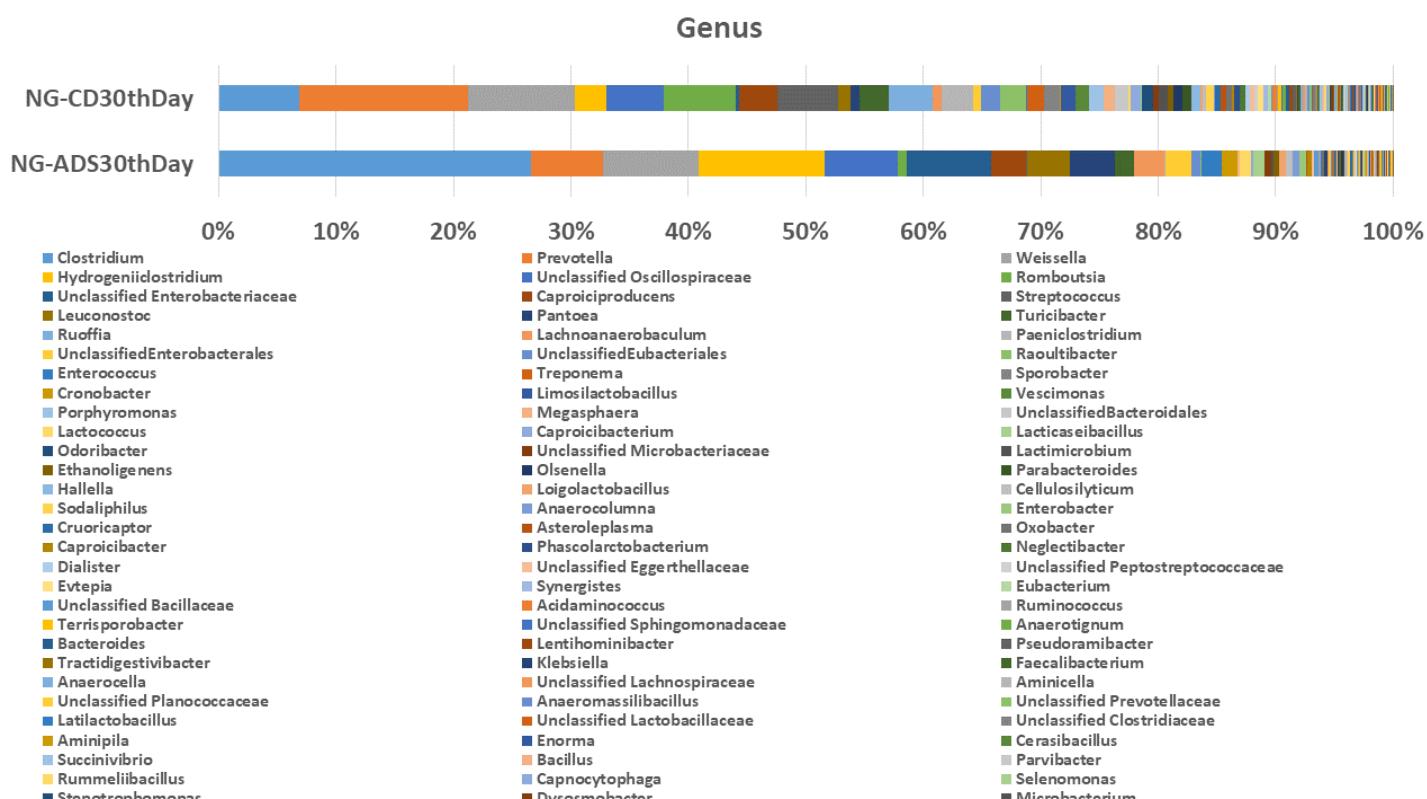


METAGENOMICS _ REPORT

METAGENOMICS REPORTFamilies Level – Taxonomy Plot Analysis



METAGENOMICS _ REPORT

METAGENOMICS REPORTGenus Level – Taxonomy Plot Analysis



Biokart India Pvt Ltd

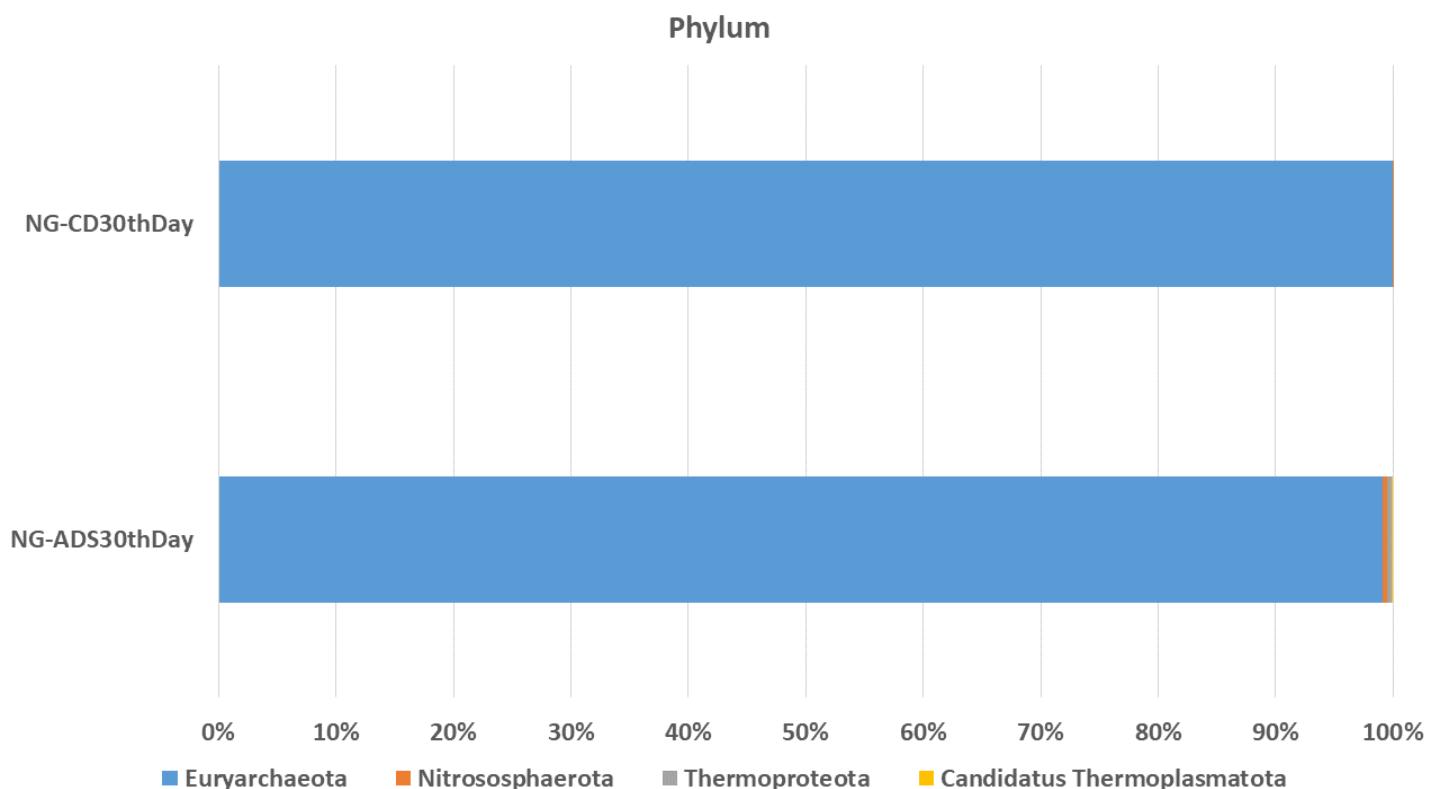
METAGENOMICS _ REPORT

METAGENOMICS REPORT

ARCHAEA - TAXONOMIC PROFILE

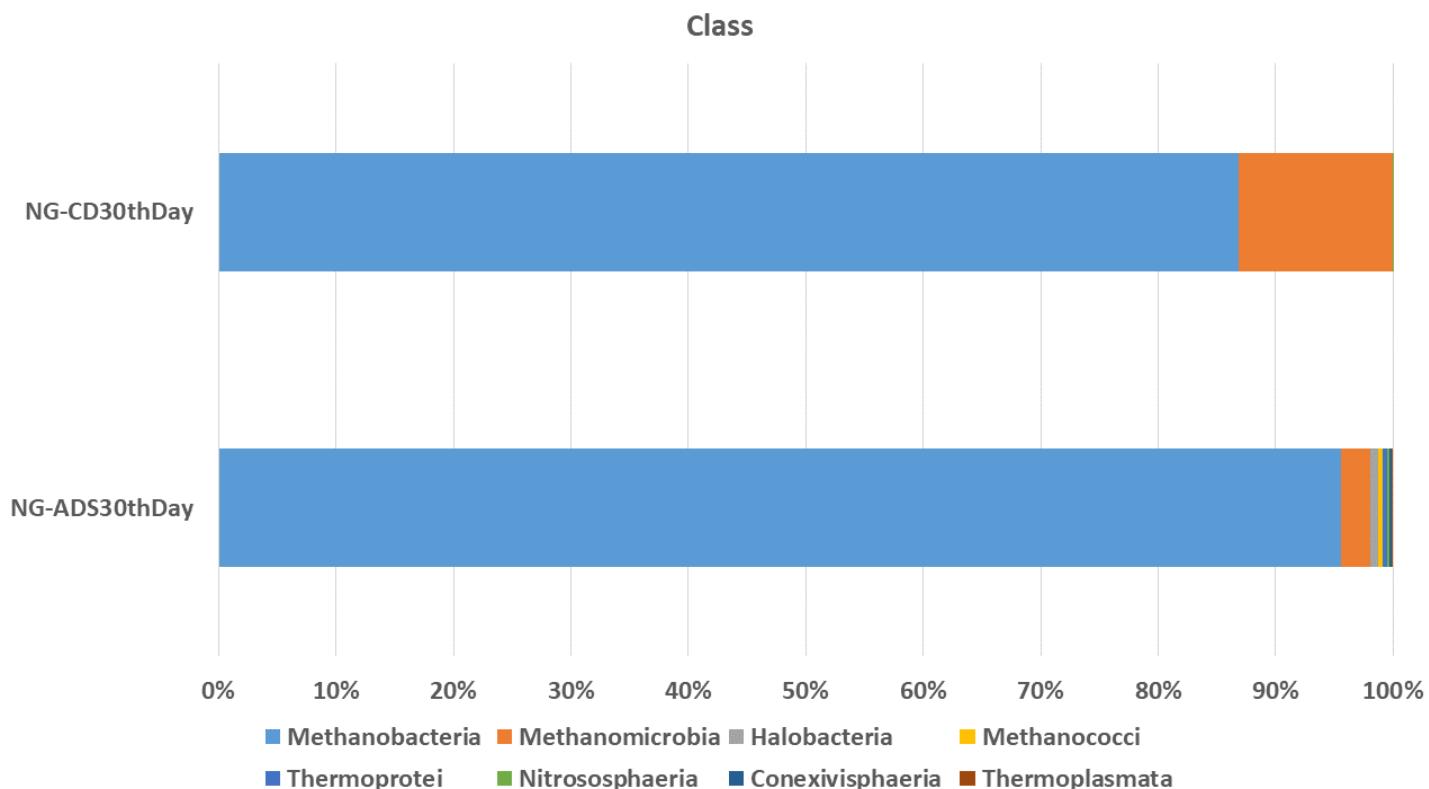


METAGENOMICS _ REPORT

METAGENOMICS REPORTPhylum Level – Taxonomy Plot Analysis

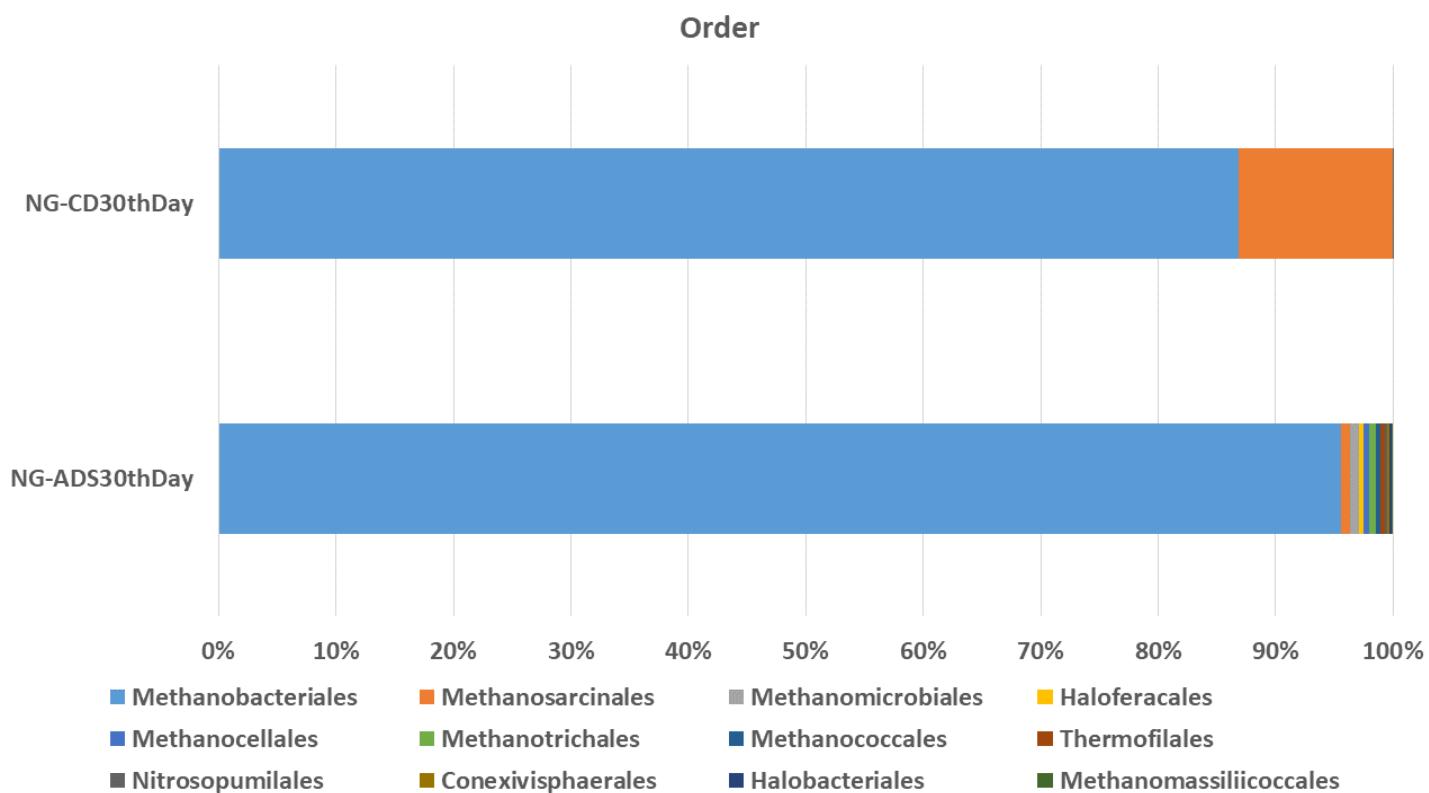


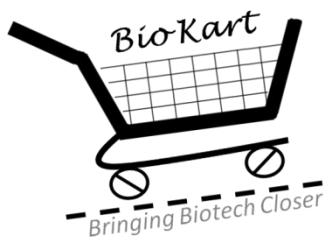
METAGENOMICS _ REPORT

METAGENOMICS REPORTClasses Level – Taxonomy Plot Analysis



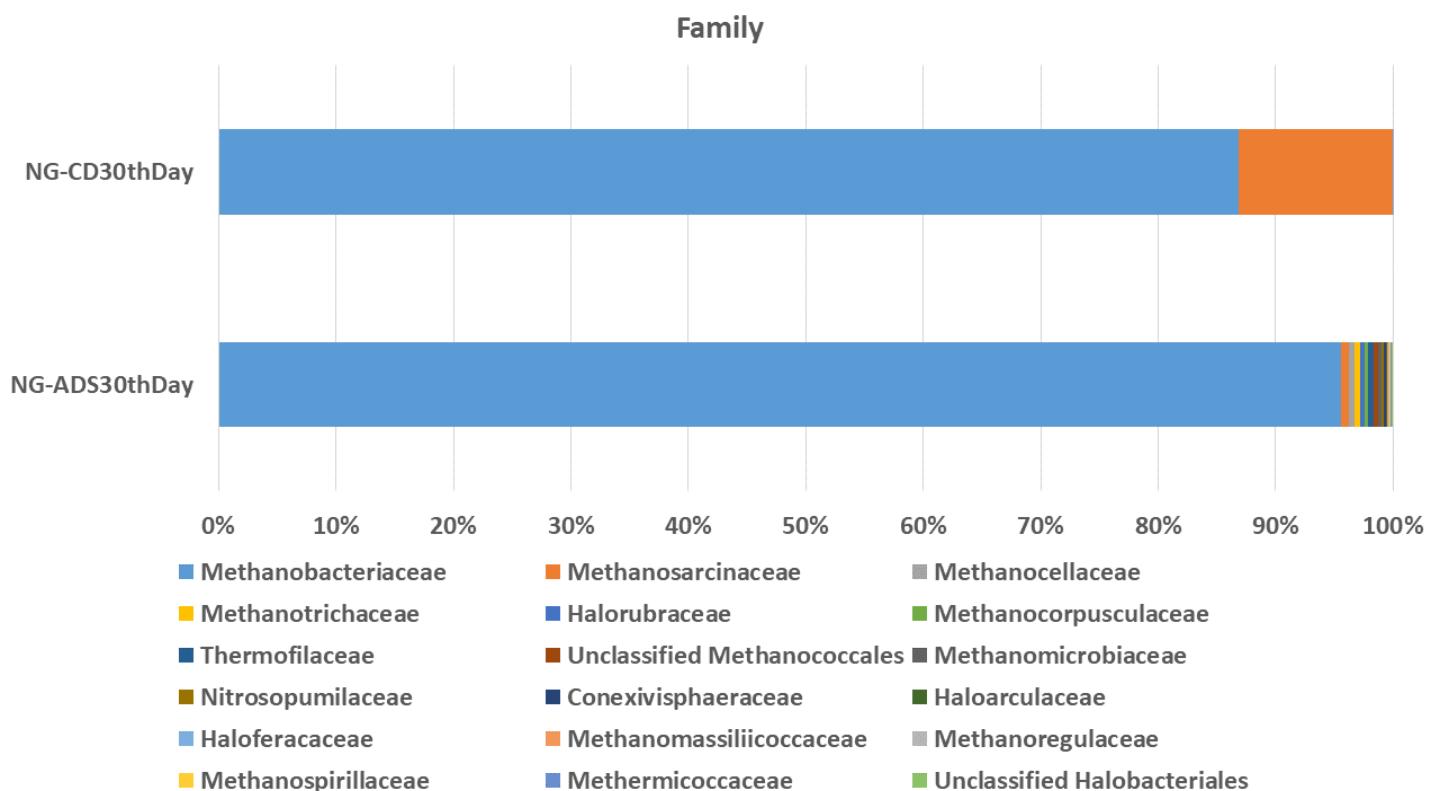
METAGENOMICS _ REPORT

METAGENOMICS REPORTOrder Level – Taxonomy Plot Analysis



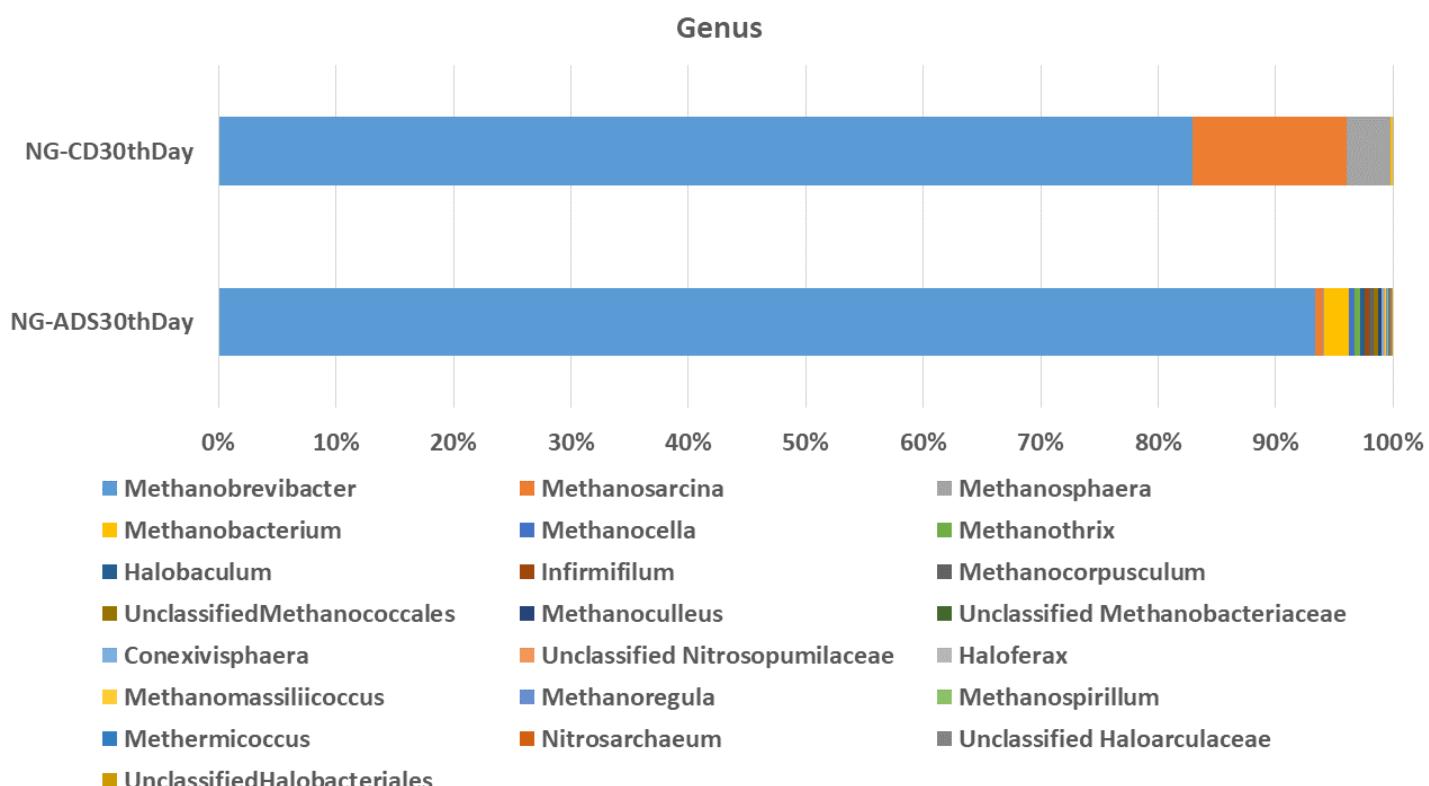
METAGENOMICS REPORT

Families Level – Taxonomy Plot Analysis





METAGENOMICS _ REPORT

METAGENOMICS REPORTGenus Level – Taxonomy Plot Analysis



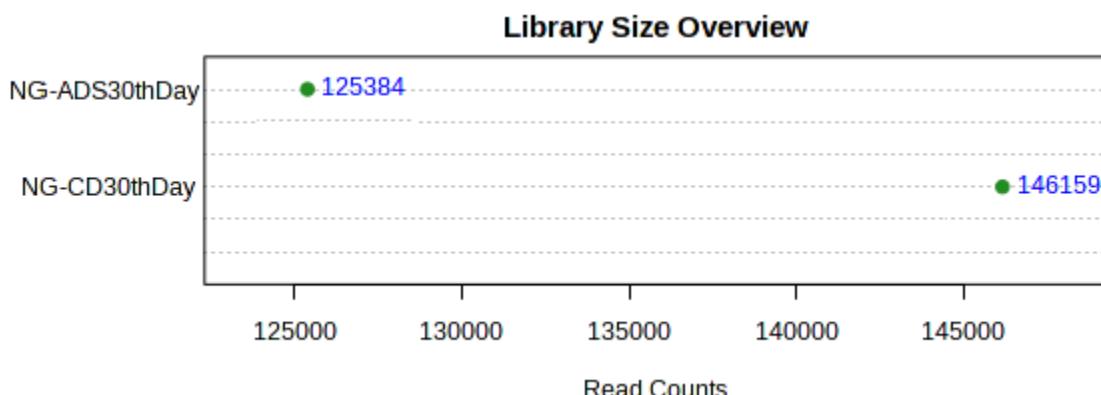
METAGENOMICS _ REPORT

METAGENOMICS REPORTData Processing

To process the metagenomics data for advanced analysis like alpha and beta diversity, the input file must contain at least 2 groups and each group must contain minimum 3 samples for the analysis,

In the analysis,

- Features with identical values (i.e. zeros) across all samples will be excluded
- Features that appear in only one sample will be excluded (considered artifacts)





METAGENOMICS REPORT

Data Filtering

Data filtering aims to remove low quality or uninformative features to improve downstream statistical analysis.

- Low count filter - features with very small counts in very few samples are likely due to sequencing errors or low-level contaminations. You need to first specify a minimum count (default 4). A 20% prevalence filter means at least 20% of its values should contain at least 4 counts. You can also filter based on their *mean* or *median* values.
- Low variance filter - features that are close to constant throughout the experiment conditions are unlikely to be associated with the conditions under study. Their variances can be measured using *inter-quantile range (IQR)*, *standard deviation* or *coefficient of variation (CV)*.

This project followed the default low count filter at a 20% prevalence with the minimum count of 4.

The low variance was filtered based on *inter-quantile range* with 10% to remove.





METAGENOMICS _ REPORT

METAGENOMICS REPORT

Data Normalization

Normalization aims to address the variability in sampling depth and the sparsity of the data to enable more biologically meaningful comparisons.

The data was rarefied to minimum library size .The data was scaled by Total sum scaling (TSS) factor to bring all the samples to the same scale.





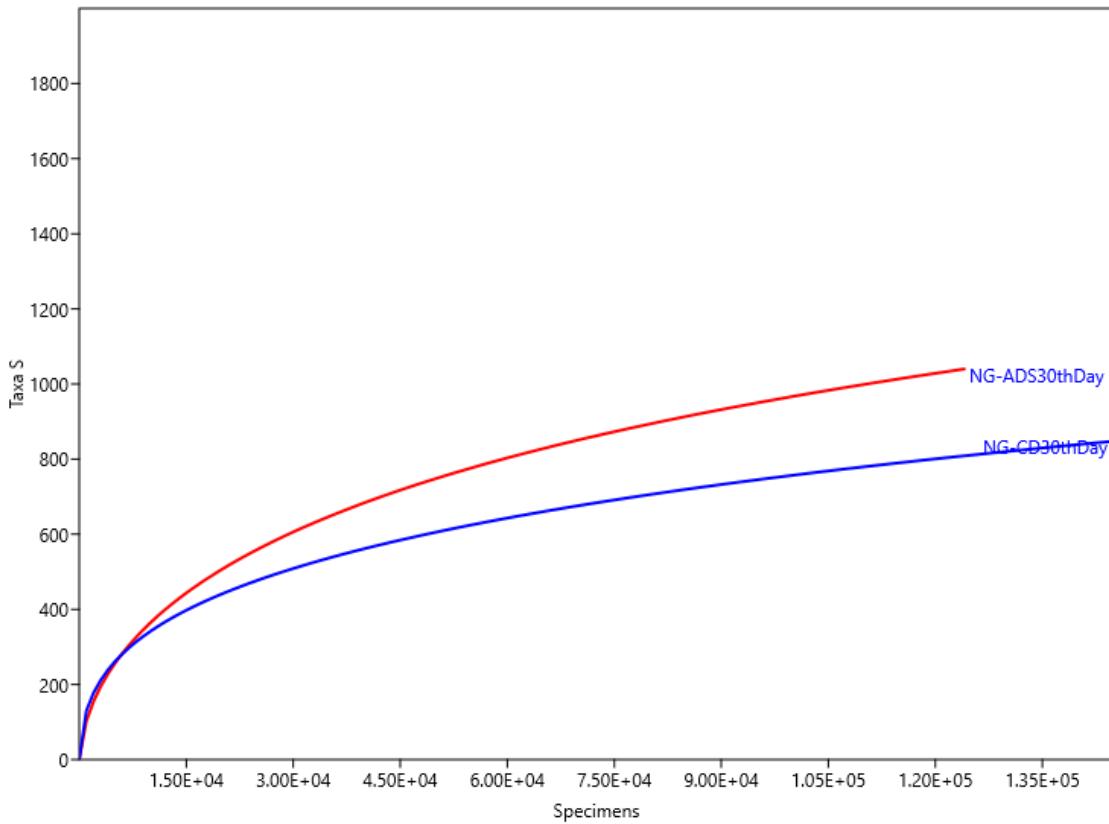
METAGENOMICS _ REPORT

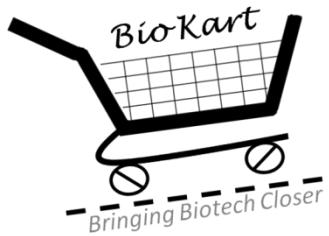
METAGENOMICS REPORT

Rarefaction Curve

All samples will be rarefied to even sequencing depth based on the sample having lowest sequencing depth.

The analysis was visualized with the filtered data source.



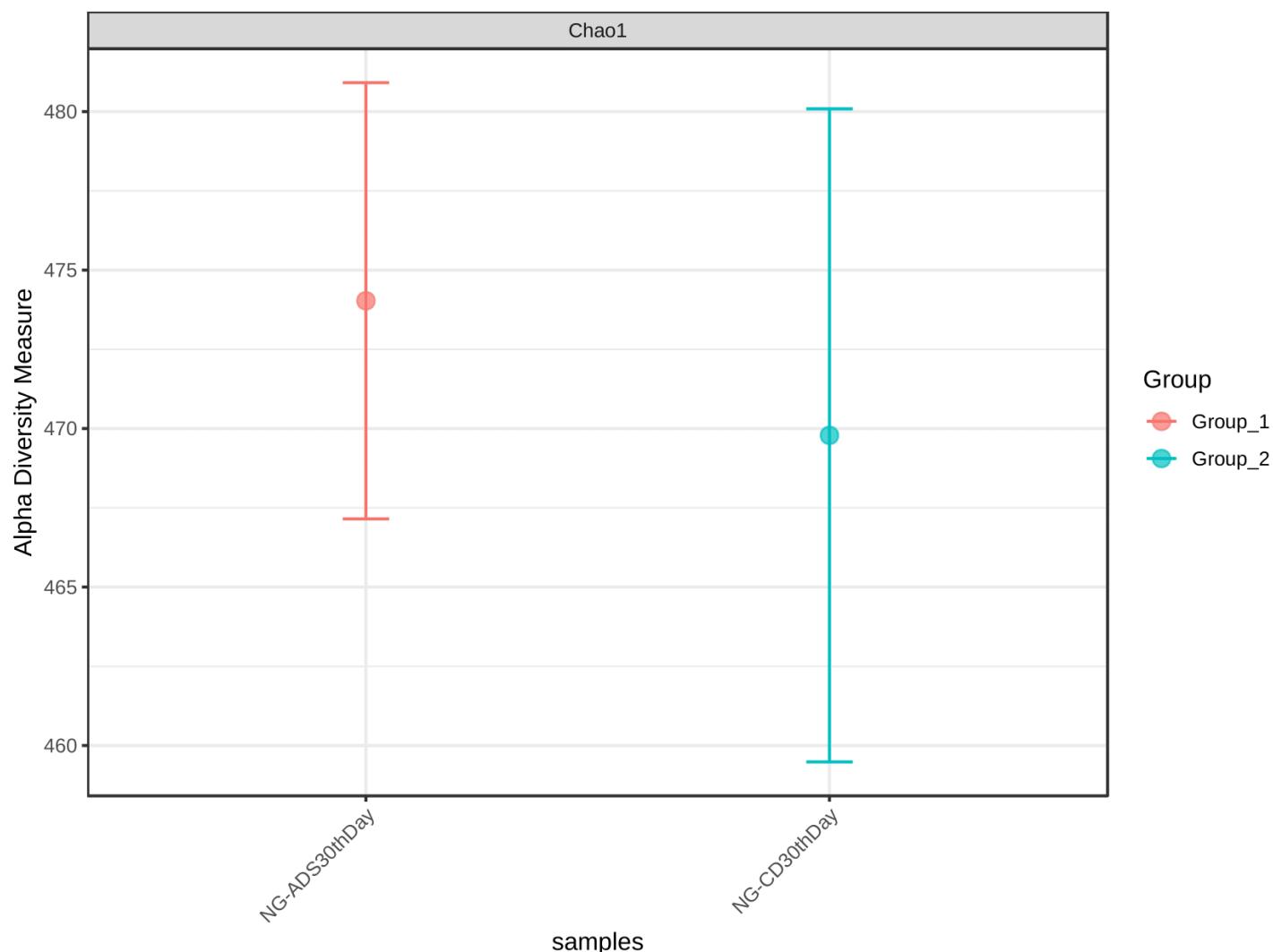


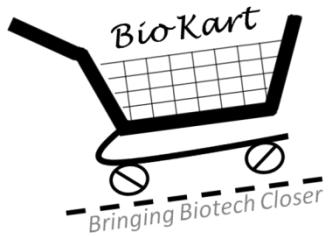
METAGENOMICS _ REPORT

METAGENOMICS REPORT

Alpha Diversity and Significance testing

The data input was filtered. And the alpha diversity was measured and resulted with 4 methods like Chao1, Shannon, Simpson and Fisher with the statistical method of T-test /ANOVA.

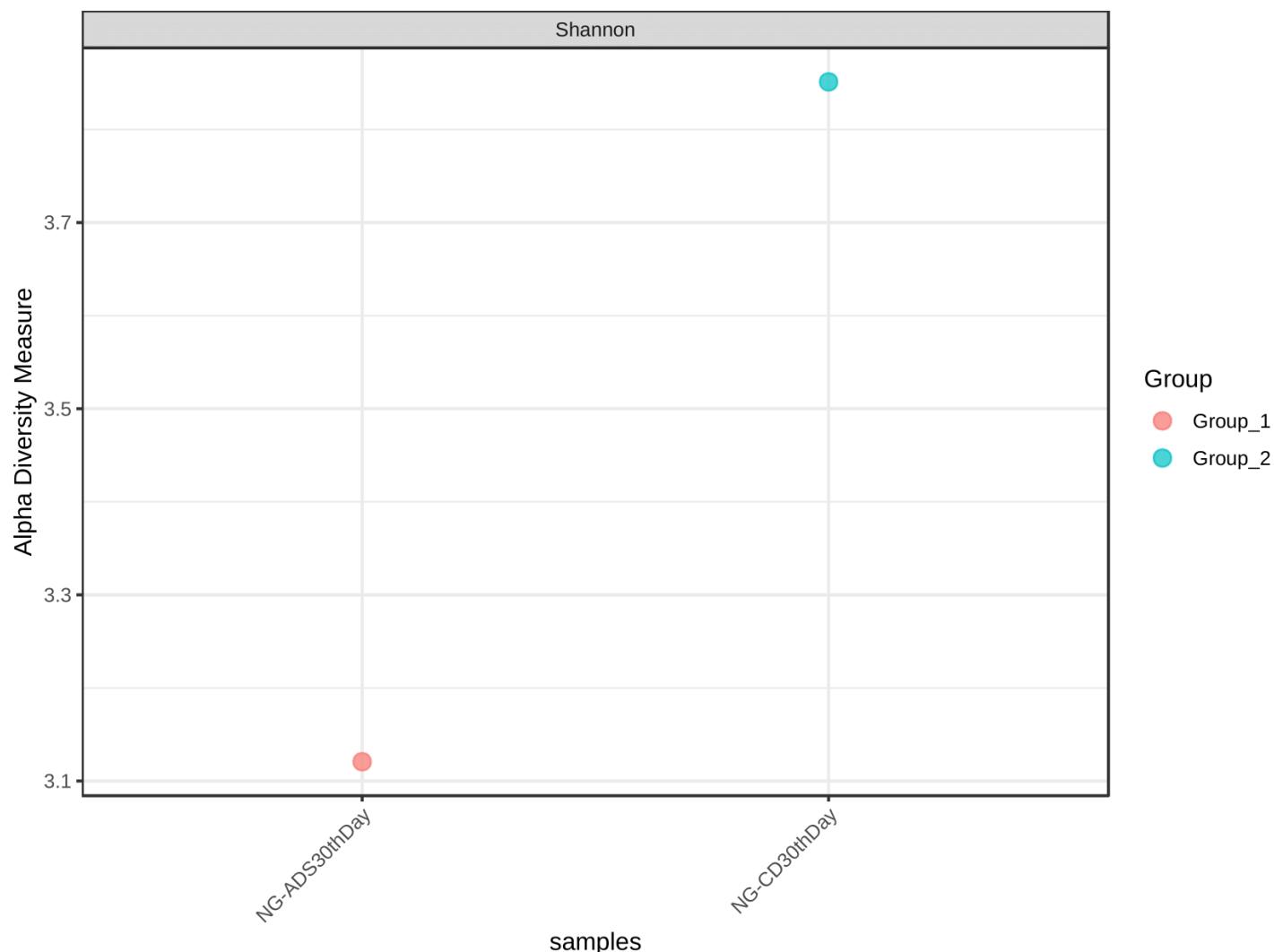


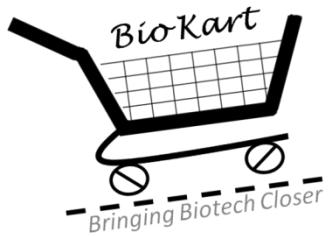


METAGENOMICS _ REPORT

METAGENOMICS REPORT
Alpha Diversity and Significance testing

The data input was filtered. And the alpha diversity was measured and resulted with 4 methods like Chao1, Shannon, Simpson and Fisher with the statistical method of T-test /ANOVA.

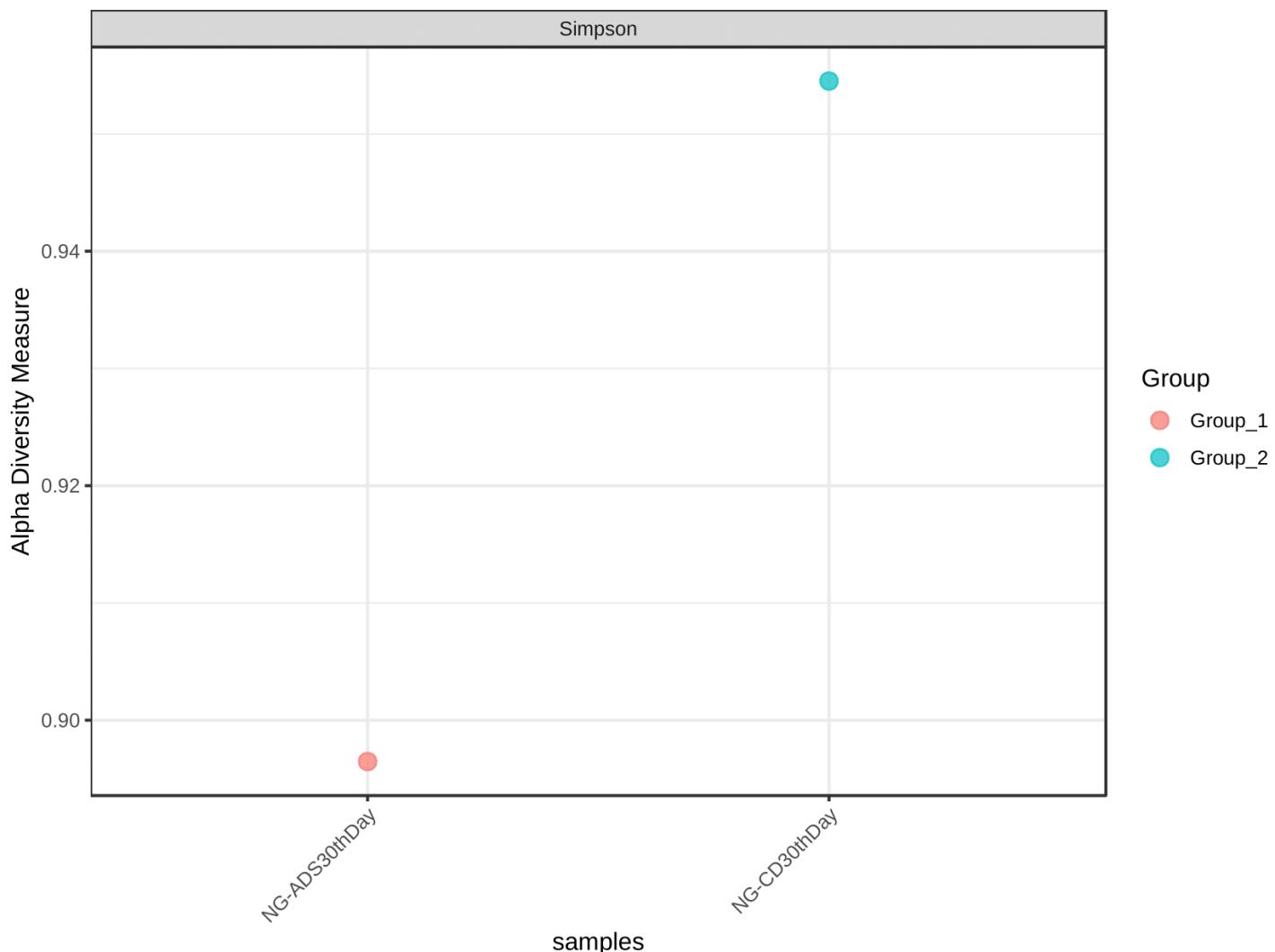


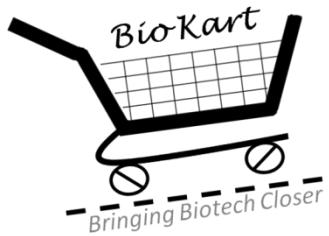


METAGENOMICS _ REPORT

METAGENOMICS REPORT
Alpha Diversity and Significance testing

The data input was filtered. And the alpha diversity was measured and resulted with 4 methods like Chao1, Shannon, Simpson and Fisher with the statistical method of T-test /ANOVA.



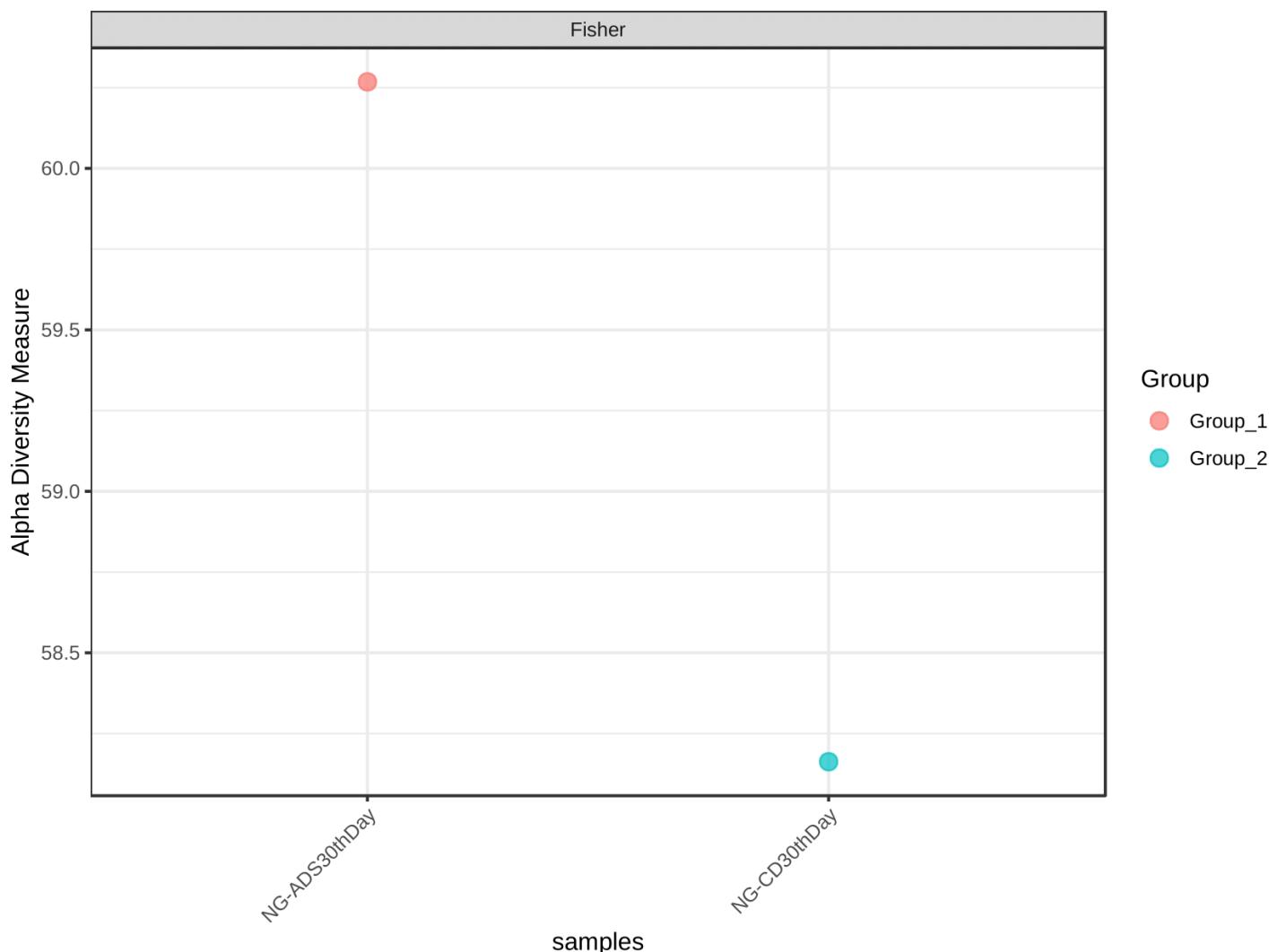


METAGENOMICS _ REPORT

METAGENOMICS REPORT

Alpha Diversity and Significance testing

The data input was filtered. And the alpha diversity was measured and resulted with 4 methods like Chao1, Shannon, Simpson and Fisher with the statistical method of T-test /ANOVA.



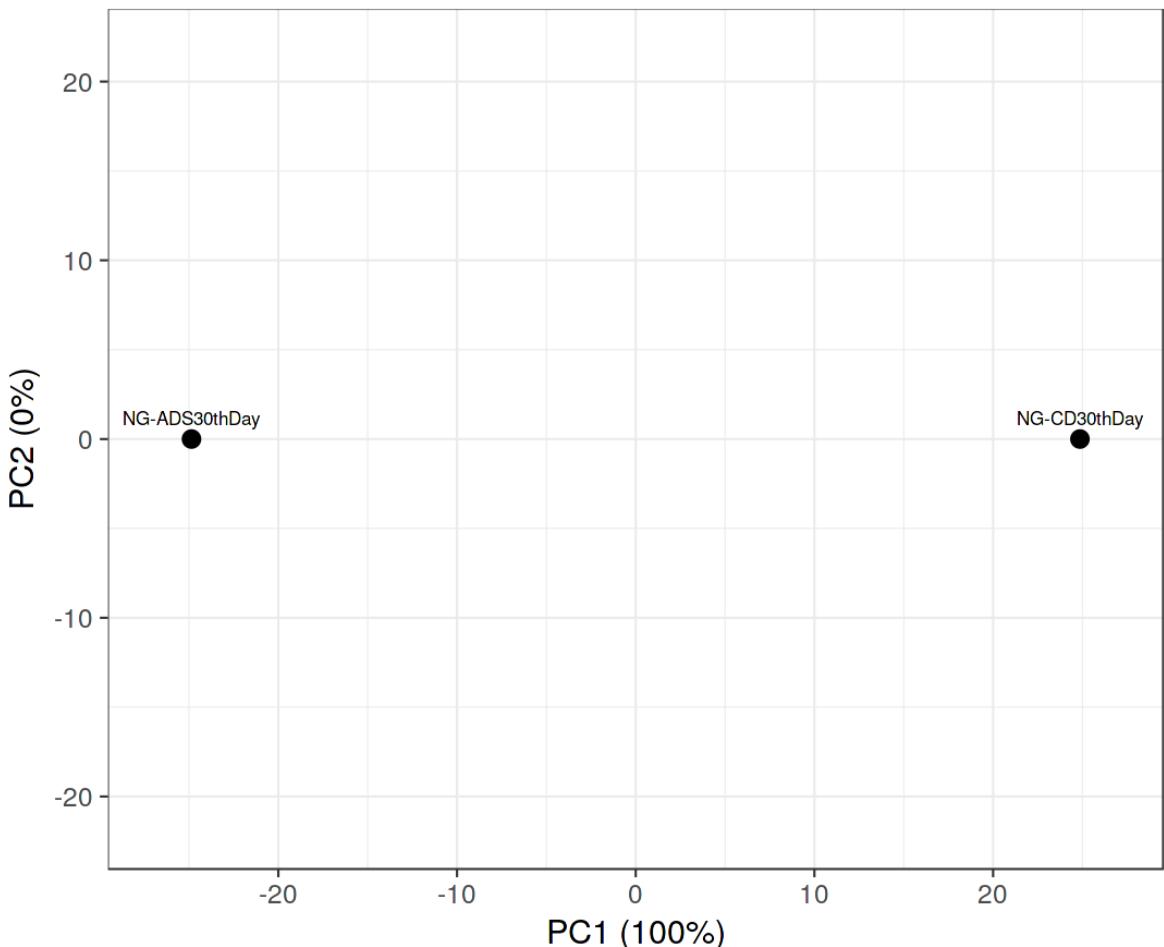


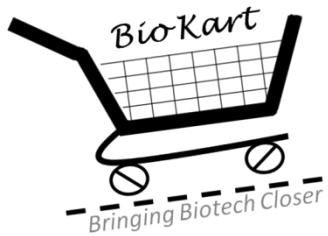
METAGENOMICS _ REPORT

METAGENOMICS REPORT

PCA Plot

Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain 0% and 100% of the total variance, respectively. N = 2 data points

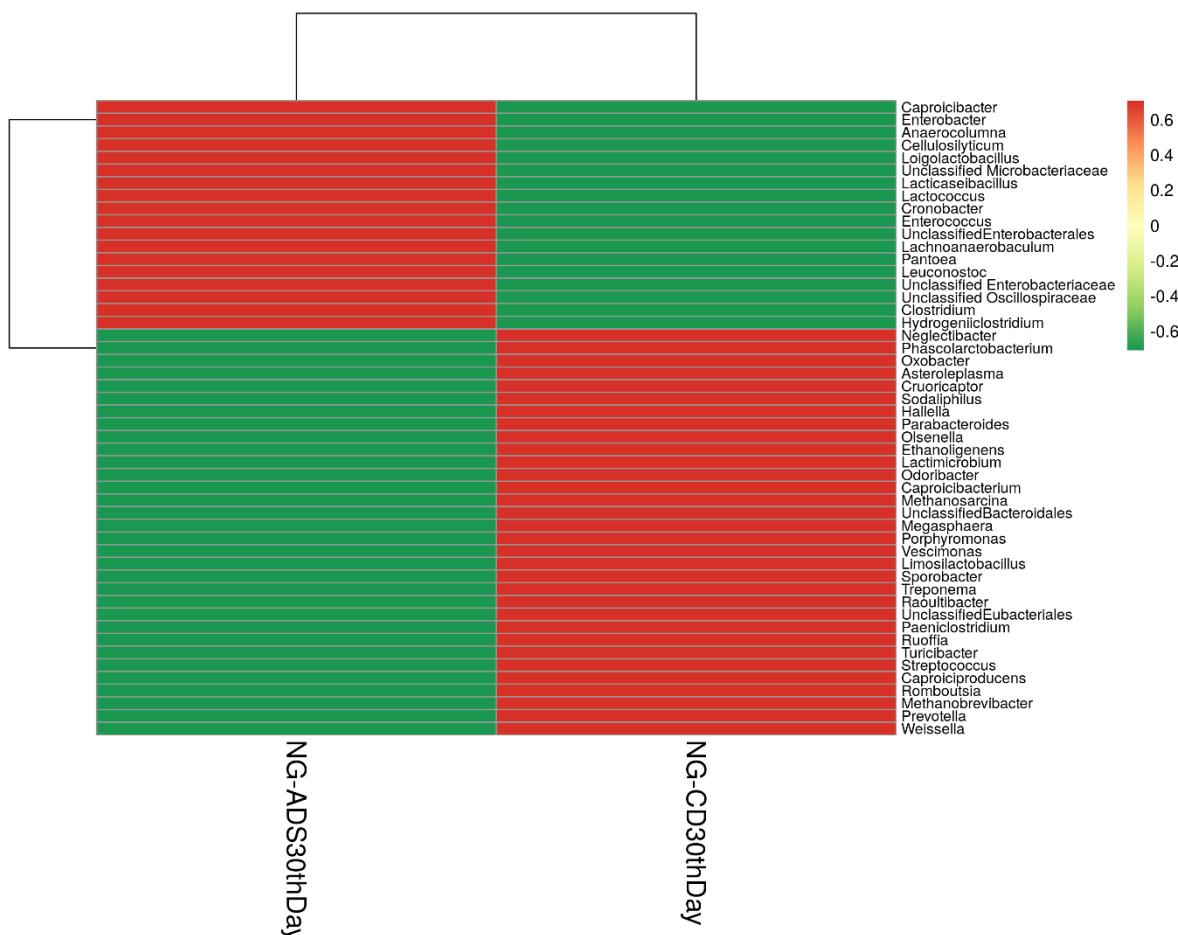


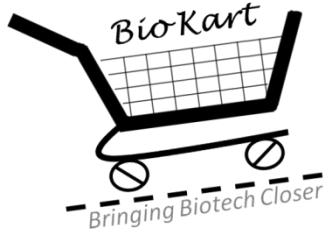


METAGENOMICS _ REPORT

METAGENOMICS REPORTHeat map clustering and visualization

Unit variance scaling is applied to sequence abundance. Both taxon rows and samples columns were clustered using correlation distance and average linkage. Top 50 organisms (genus level) were taken for the heatmap construction.

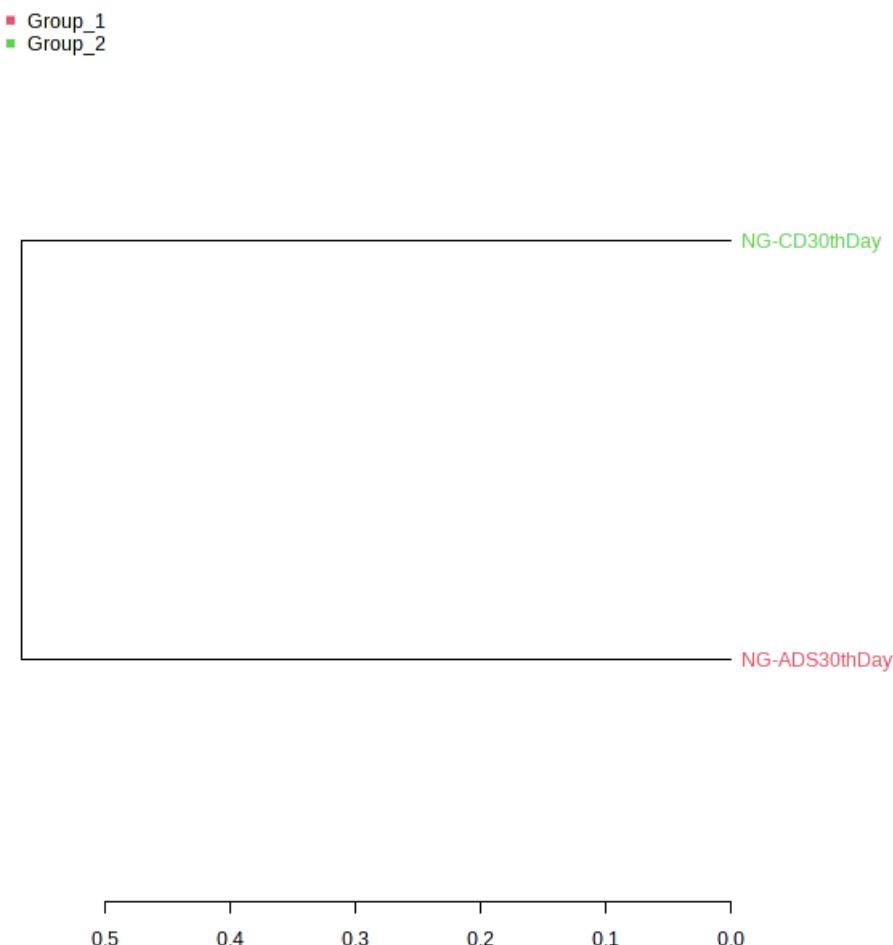




METAGENOMICS _ REPORT

METAGENOMICS REPORTDendrogram

The dendrogram was constructed based on the distance measure of Bray-Curtis Index with Ward clustering algorithm.

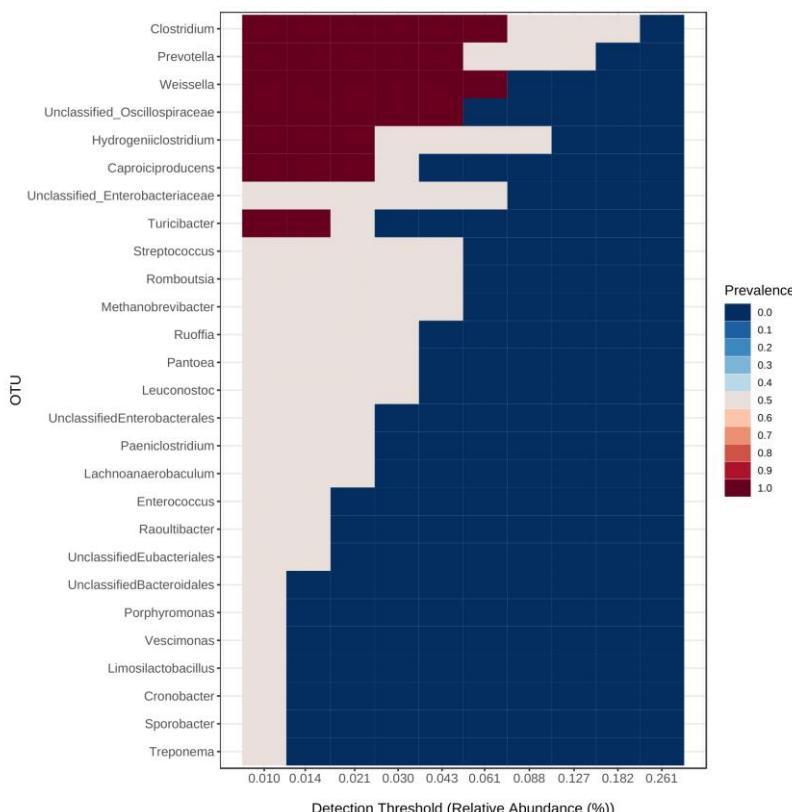




METAGENOMICS _ REPORT

METAGENOMICS REPORTCore Microbiome

The core microbiome refers to the set of taxa that are detected in a high fraction of the population above a given abundance threshold. The count data is transformed to compositional (relative) abundance in order to perform such analysis.

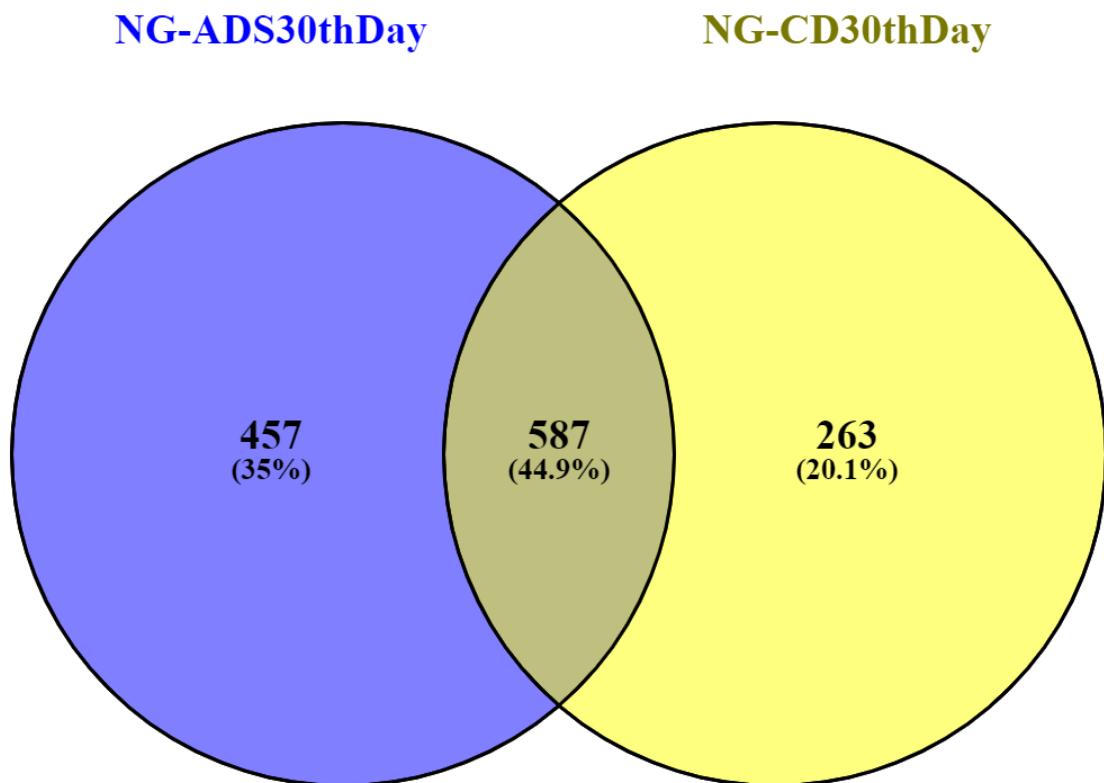


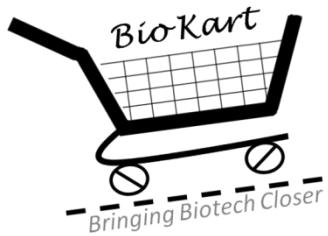
The data was visualized with the sample prevalence of 20% and relative abundance of 0.01 %.



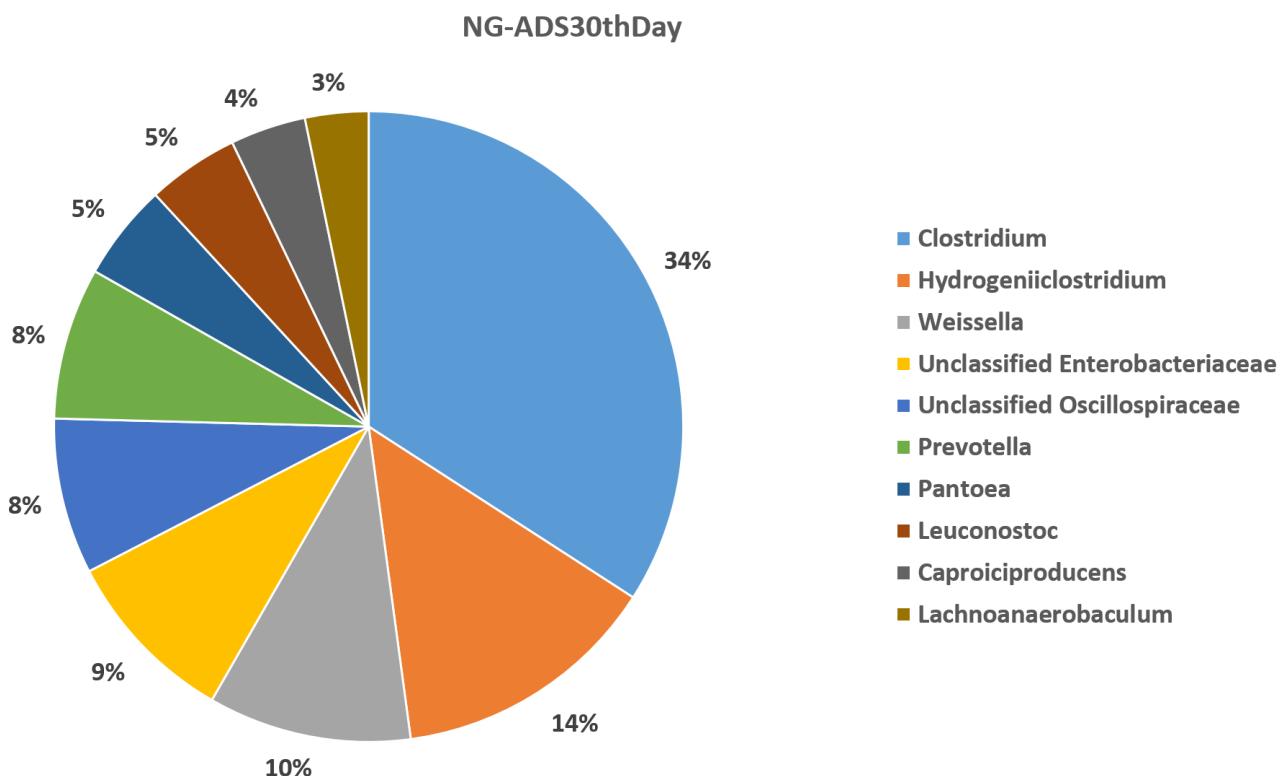
METAGENOMICS REPORT

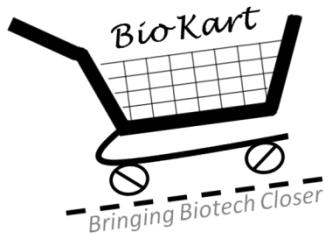
Venn Diagram



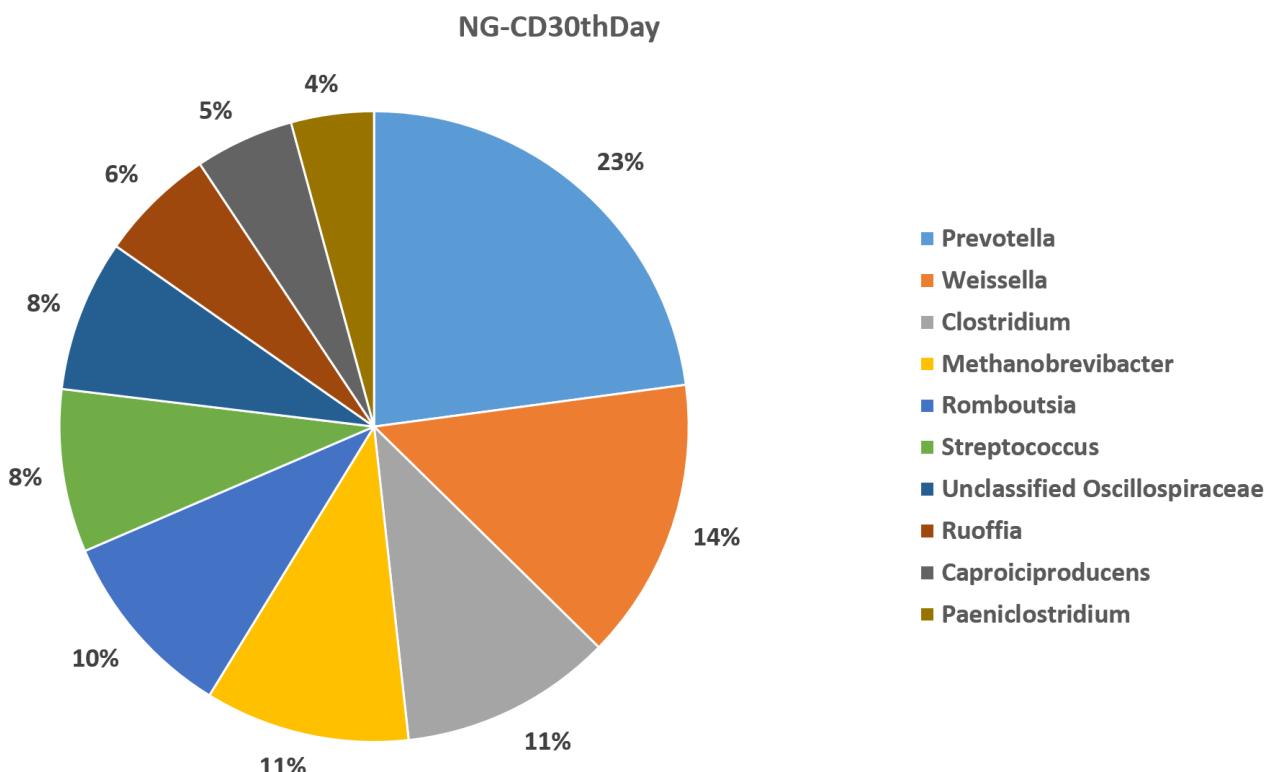


METAGENOMICS _ REPORT

METAGENOMICS REPORTTop 10 Enrichment GENUS level



METAGENOMICS _ REPORT

METAGENOMICS REPORTTop 10 Enrichment GENUS level



Biokart India Pvt Ltd

TIN: 29400662787

PAN: AAECB8426A

CIN: U74900KA2011PTC061787

Additional Documents Provided:

- 1) High Quality Images
- 2) OTU table
- 3) Related tables
- 4) Comparison pdf

END OF REPORT

Checked by,

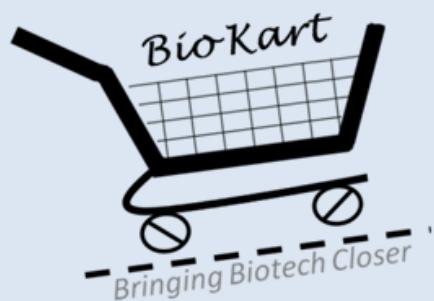
Bioinformatician
Biokart India Pvt. Ltd.

Biokart India Pvt. Ltd.,

5M-612, 5th Main Road, OMBR Layout, Bengaluru, Karnataka 560043

Phone: +91-90084-91839 Email: info@biokart.com Website: www.biokart.com





Biokart India Pvt. Ltd.

Bringing Biotech Closer

TIN: 29400662787

PAN: AAECB8426A

CIN: U74900KA2011PTC06DDSKVG1387