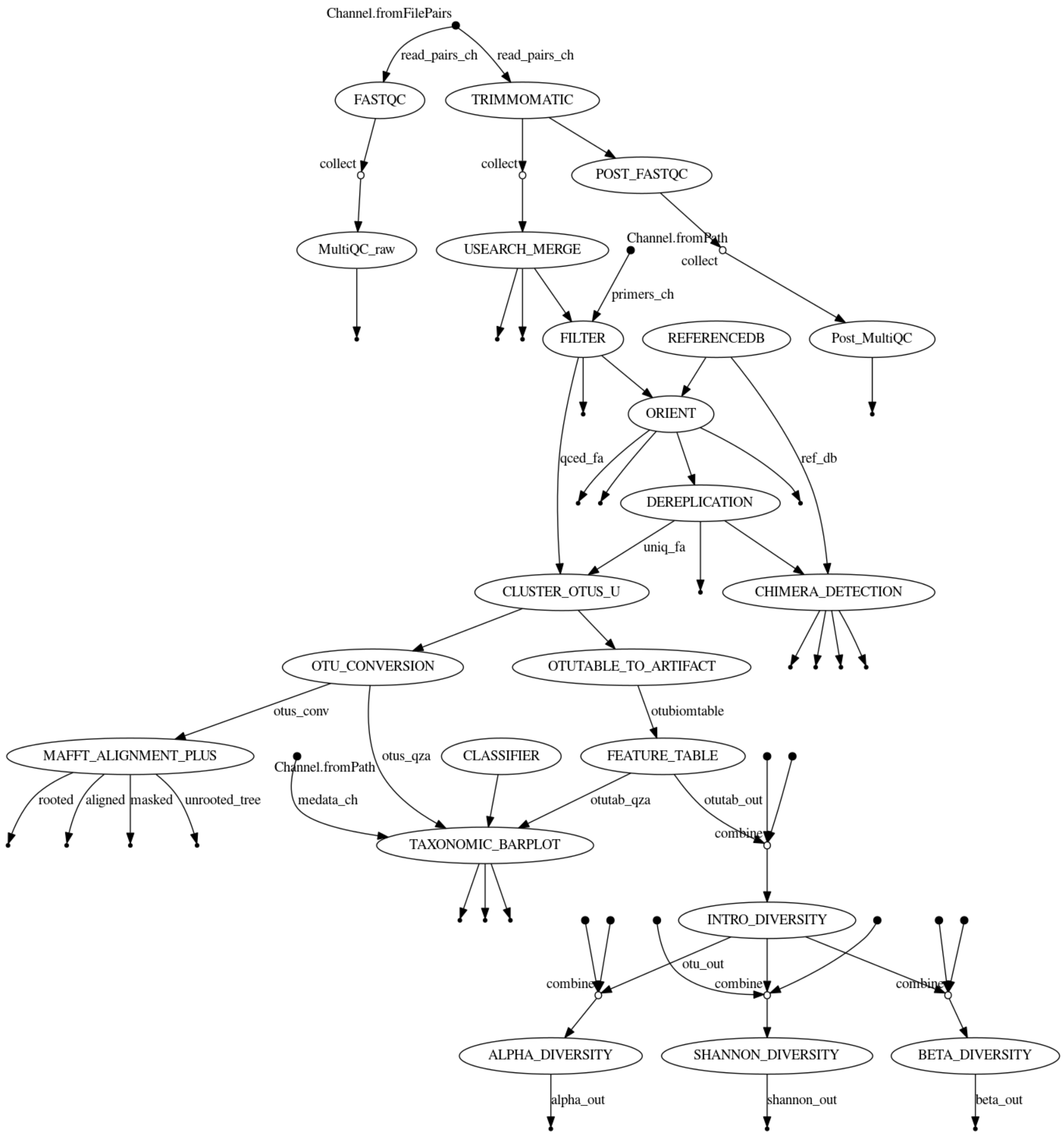
Qiime Nexflow Pipeline

We developed a Qiime2 16s nextflow workflow utilizing the following tools:

Tool	*Version*
fastqc	v0.11.9
multiqc	v1.4
trimommatic	v0.39
usearch	v11.0
vsearch	v2.16
qiime2 env	2020.8
nextflow	20.10.0 (dsl 2)

Our analysis pipeline follows the following order: 1. Quality check of raw reads 2. Trimming of adapters from reads 3. Merging/Stitching 4. Filtering and Primer removal 5. Orientation 6. Dereplication 7. Chimera detection 8. Clustering OTUs 9. Phylogeny 10. Taxonomy 11. Alpha diversity and Beta diversity

This workflow has been summarized in the Nextflow analysis figure below.



Sample info

We had a total of 124 paired end reads samples. These samples had a length of 250bp.

Runtime analysis

The total runtime of each process is summarized in this Nextflow report. The timeline report shows the job wall time and memory used by each individual sample in the different steps. It also provides info on the total time used to run the entire workflow.

A summary of this information in the form of the average time for each step, the percentage of CPUs used, and memory available in this report.

The average storage used by the different steps is summarized below;

Analysis Step	time	Storage
Quality Check (fastqc & multiqc)		388M & 4.5M
Trimming		21G
Quality Check (fastqc & multiqc)		388M & 4.5M
Merging (usearch)		14G
Filtering (vsearch)		4.6G
Orient (vsearch)		4.9G
Dereplication (vsearch)		197M
Chimera detection (vsearch)		261M
Cluster OTUs (usearch)		792K
Reference DB		7.2M
Artifacts and classifier		28M
Visualization (qiime2)		6.1M

Quality check

The quality of the read was checked using fastqc. The data was characterized by low per base sequence content, high sequence duplication, and overrepresntation.

A summary for all the samples can be seen in this muiltiqc report

Trimming

We trimmed using trimmomatic to remove sequencing adapters, and low quality reads. The following parameters were used to improve the read quality: - phred score 33 - min length 36 - sliding window 4:15

Post trimming quality check

A summary of the results post trimming is available in this multiqc report

Merging

Using `usearch` merge, we were able to stitch 91.76%. Unstitched reads were discarded.

```
Totals:
  20578230  Pairs (20.6M)
  18883320  Merged (18.9M, 91.76%)
  11799125  Alignments with zero diffs (57.34%)
    339274  Too many diffs (> 10) (1.65%)
     67847  Fwd too short (< 64) after tail trimming (0.33%)
    621679  Rev too short (< 64) after tail trimming (3.02%)
    666110  No alignment found (3.24%)
         0  Alignment too short (< 16) (0.00%)
         0  Merged too short (< 16)
         0  Merged too long (> 486)
     66970  Staggered pairs (0.33%) merged & trimmed
    184.49  Mean alignment length
    300.18  Mean merged length
      0.40  Mean fwd expected errors
      0.61  Mean rev expected errors
      0.22  Mean merged expected errors
```

Filtering

vsearch was used with the following parameters:

```
vsearch -fastq_filter all_reads_merged.fastq \
    --fastq_maxee 1.0 \
    --fastq_stripleft 24 \
    --fastq_stripright 25 \
    --fastq_qmax 75 \
    --fastaout filtered.fasta \
    --log filter.log
```

The `fastq_stripleft` and `fastq_stripright` are the lengths used to trim forward and reverse primers, respectively. The `fastq_qmax` is the maximum quality score to use.

From the above, 18128002 sequences were kept (of which 18128002 truncated) and discarded755318 sequences.

Orientation

```
7438266 nt in 5181 seqs, min 1205, max 1585, avg 1436

Forward oriented sequences: 17753437 (97.93%)
Reverse oriented sequences: 53738 (0.30%)
All oriented sequences:     17807175 (98.23%)
Not oriented sequences:     320827 (1.77%)
Total number of sequences:  18128002
```

Dereplication

```
4491530932 nt in 17805181 seqs, min 32, max 436, avg 252
minseqlength 32: 1994 sequences discarded.

758165 unique sequences, avg cluster 23.5, median 1, max 2501007
```

Chimera detection

```
7438266 nt in 5181 seqs, min 1205, max 1585, avg 1436

    0.28  minh
    8.00  xn
    1.40  dn
    1.00  xa
    0.80  mindiv
    0.55  id
       2  maxp
```

```
uniqs.fasta: 114201/758165 chimeras (15.1%)
```

Cluster ASVs

We used the `usearch unoise3` algorithm to do clustering, which has the following conditions.

1. ASVs that are more than 97% identical are clustered together
2. Sequences with less than eight counts are discarded. Bokulich et al. suggest using a 0.005% minimum abundance threshold, which in our case came out to be ~9, that is what we specified.
3. Sequences are ranked based on abundance, and singletons are discarded
4. Chimeric reads are discarded

With these conditions, the number of ASVs generated is lower compared to the Dada2 pipeline. The advantage of `unoise3` is that it doesn't produce spurious ASVs, which can be an issue in Dada2. It also shows the best balance between resolution and specificity. These observations have been previously made from comparisons of pipelines, seen here and here.

1760 Unique Sequences were detected using `unoise3`. The counts table contains 1493 ASVs entries and counts for each sample.

```
# Cluster Totals from Unoise3
1760 good, 4228 chimeras

# Counts table
376414 nt in 1760 seqs, min 32, max 407, avg 214
Masking 100%
Counting k-mers 100%
Creating k-mer index 100%
Searching 100%
Matching unique query sequences: 16998774 of 18128002 (93.77%)
```

Phylogeny

Alignment was done using `mafft` followed by masking non-informative sites. The phylogenetic tree was constructed using `fasttree`. The rooted ASVs tree can be visualized below.

Taxonomy

The Greengenes database was used for the classification of the ASVs. The classification presented below is up to level 3.

BV negative samples were characterized mainly by *Lactobacillales* species with relative abundance >50% for most samples, as shown below.

Low inflammation status was majorly characterized with *Lactobacillales* in most samples, as seen below.

Alpha and Beta Diversity

Bray Curtis Principal Coordinate Analysis (PcoA)

The outcome of a PCoA tells us that objects ordinated closer to one another are more similar than those ordinated further away. Samples positive for BV cluster together while those negative for BV also cluster separately.

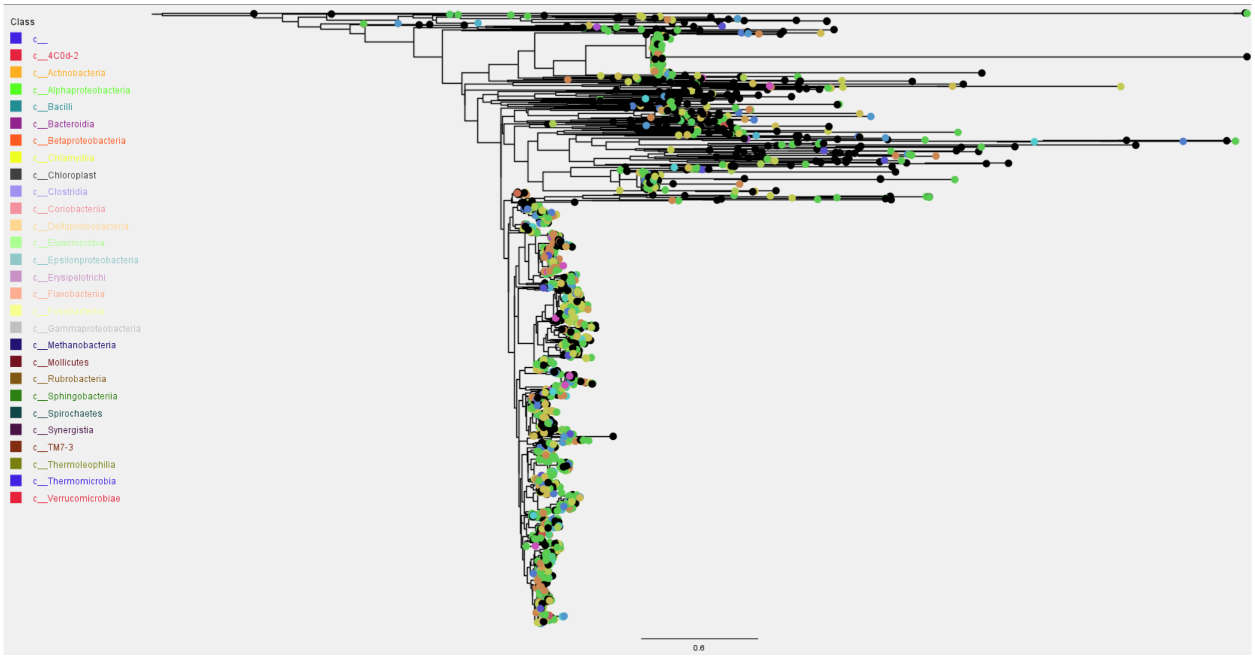


Figure 1: Tree

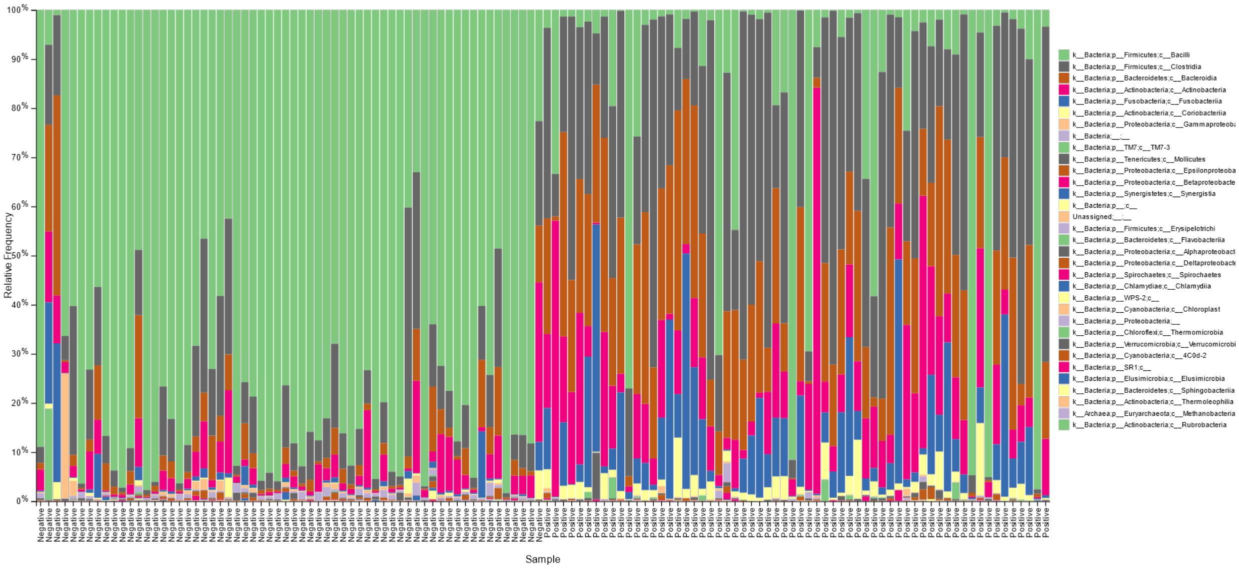


Figure 2: BV status

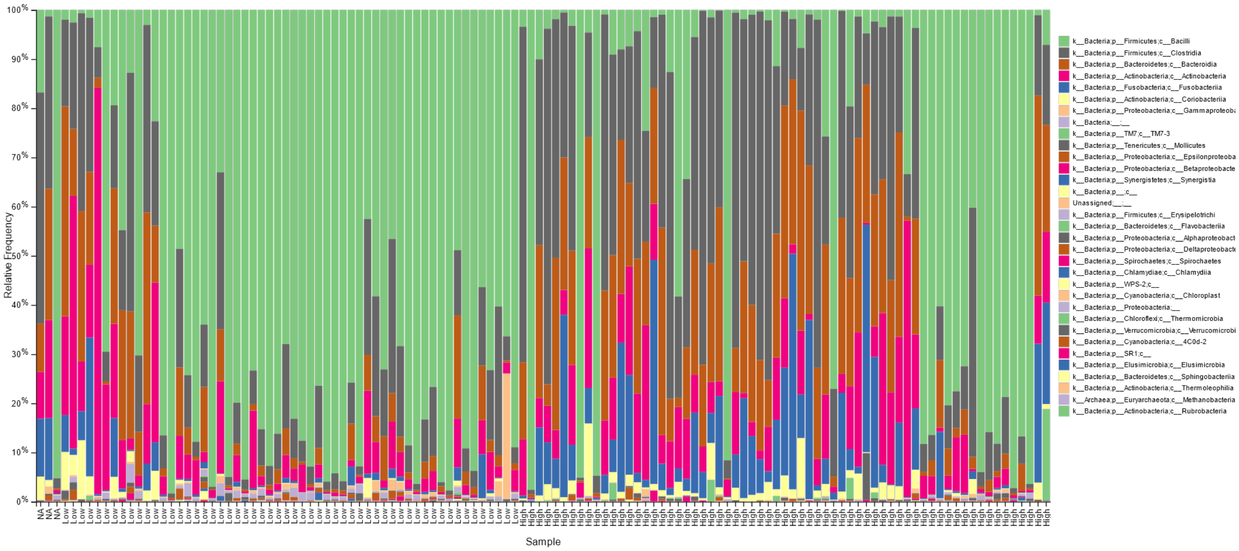
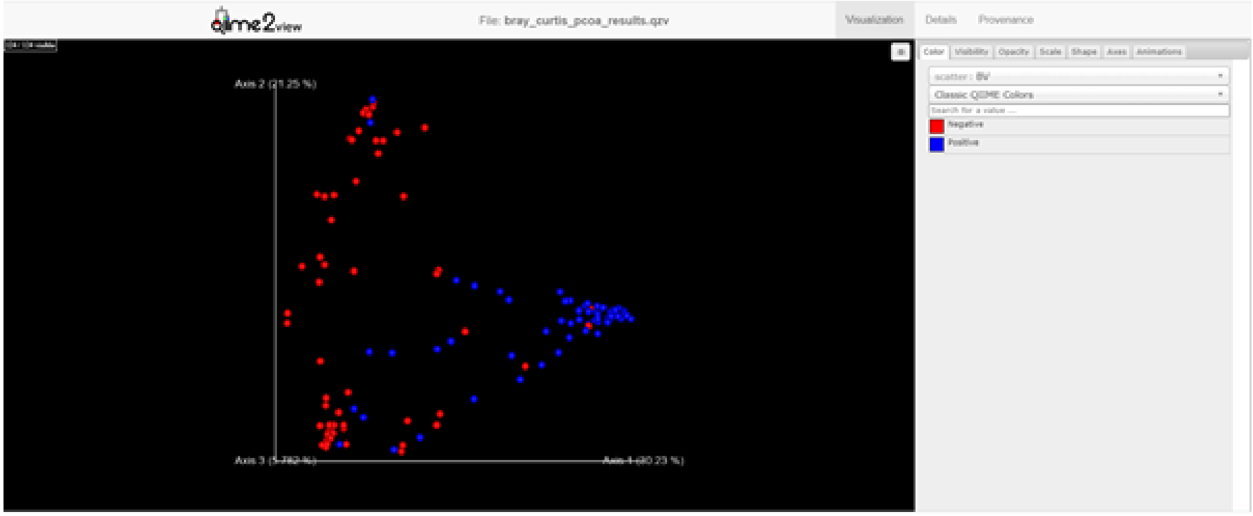


Figure 3: Inflamation status



File: bray_curtis_pcoa_results.qzv

scatter : BV

Classic QIIME Colors

🟥 Negative

🟦 Positive

Figure 4: BV status PcoA

In the figure below, samples with high inflammation cluster together, those with low inflammation cluster together, while those that are neutral cluster together separately.

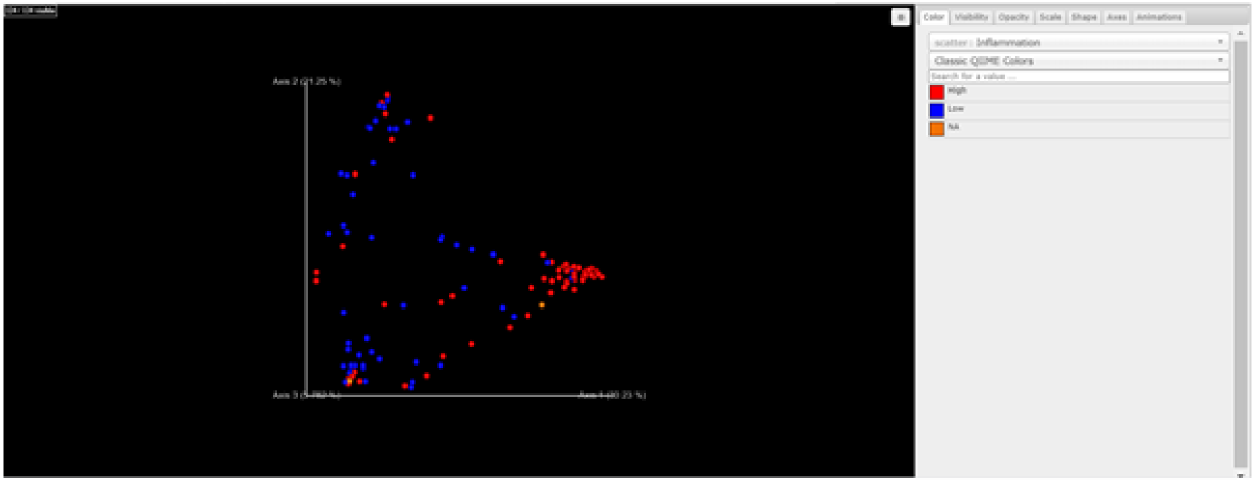


Figure 5: Inflammation status PCoA

The samples also cluster based on the Body Mass Index (BMI) as shown below:

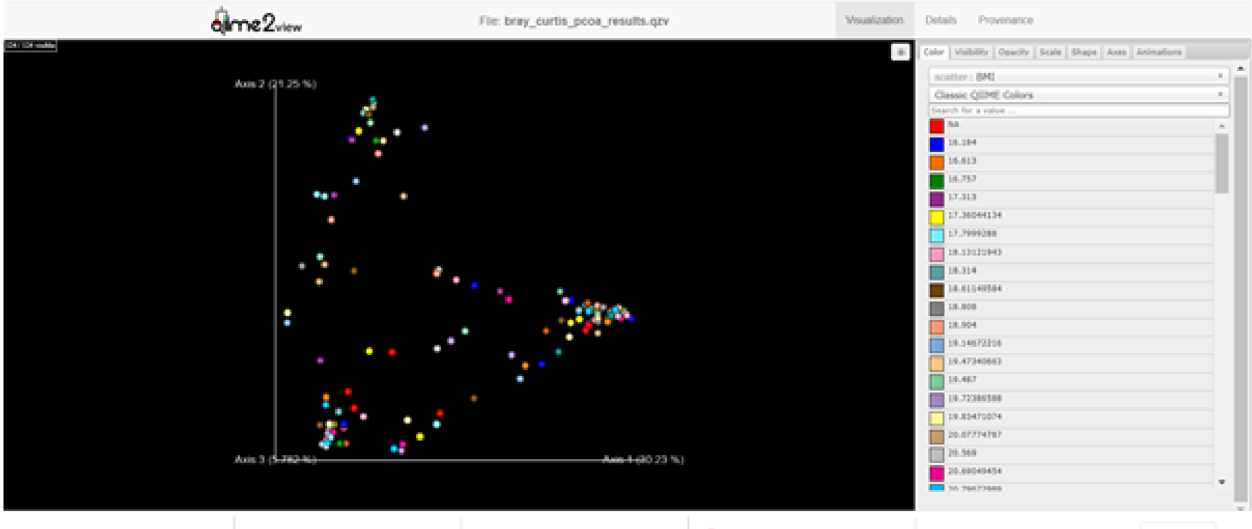


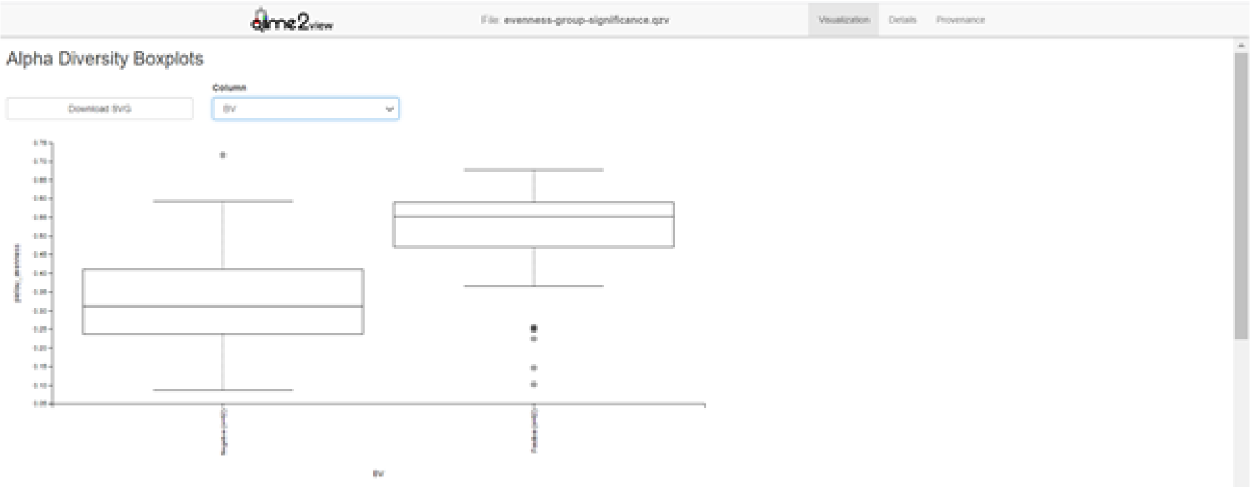
Figure 6: BMI status PCoA

Evenness group significance

Evenness refers to how well the taxa are distributed within samples, that is, their abundances. The figure below shows us the abundance of samples based on different parameters:

Samples positive for BV and those negative for BV.

Samples with high inflammation and low inflammation

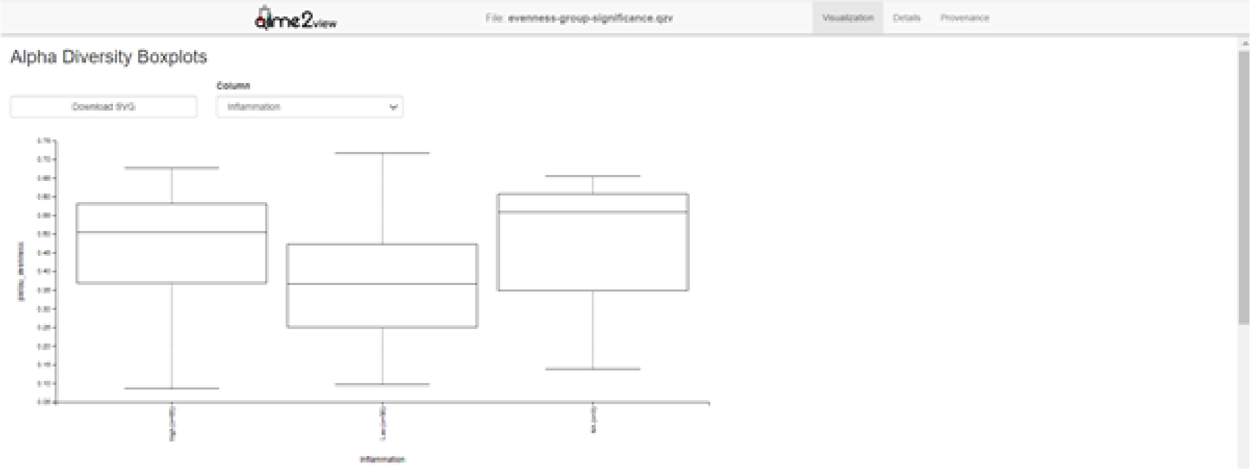


File: evenness-group-significance.qzv

Alpha Diversity Boxplots

Column: BV

Figure 7: BV Evenness

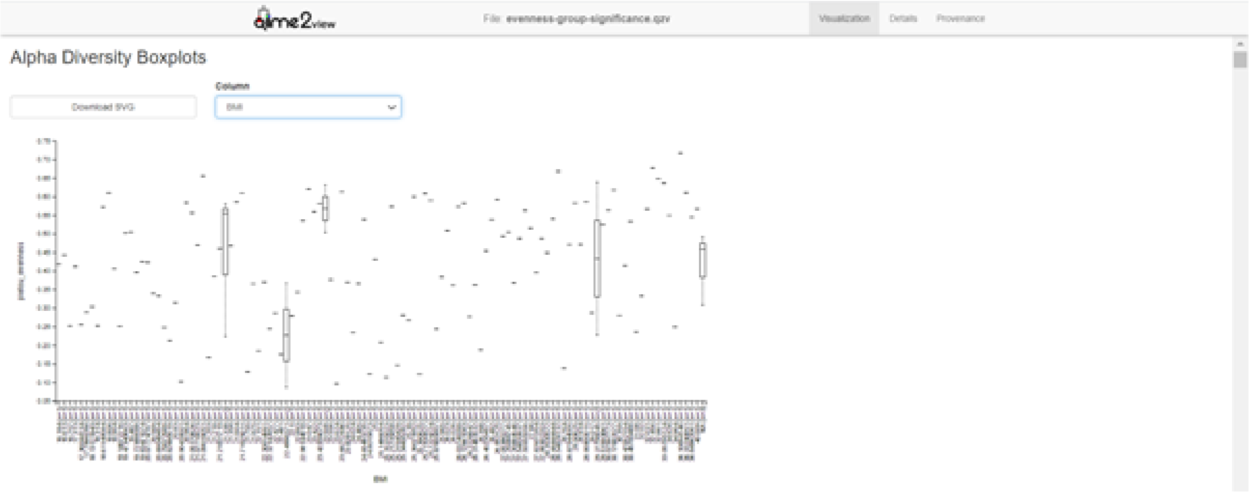


File: evenness-group-significance.qzv

Alpha Diversity Boxplots

Column: Inflammation

Figure 8: Inflammation_evenness



File: evenness-group-significance.qzv

Alpha Diversity Boxplots

Column: BMI

Figure 9: BMI_Evenness

Sample abundance based on BMI

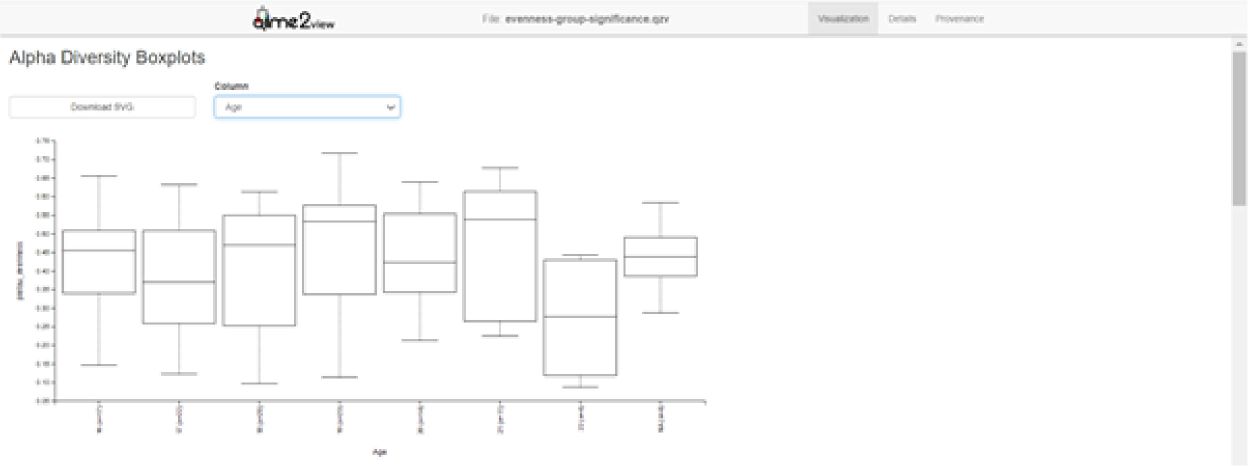


Figure 10: Age_Evenness

Sample abundance based on age

Shannon group significance

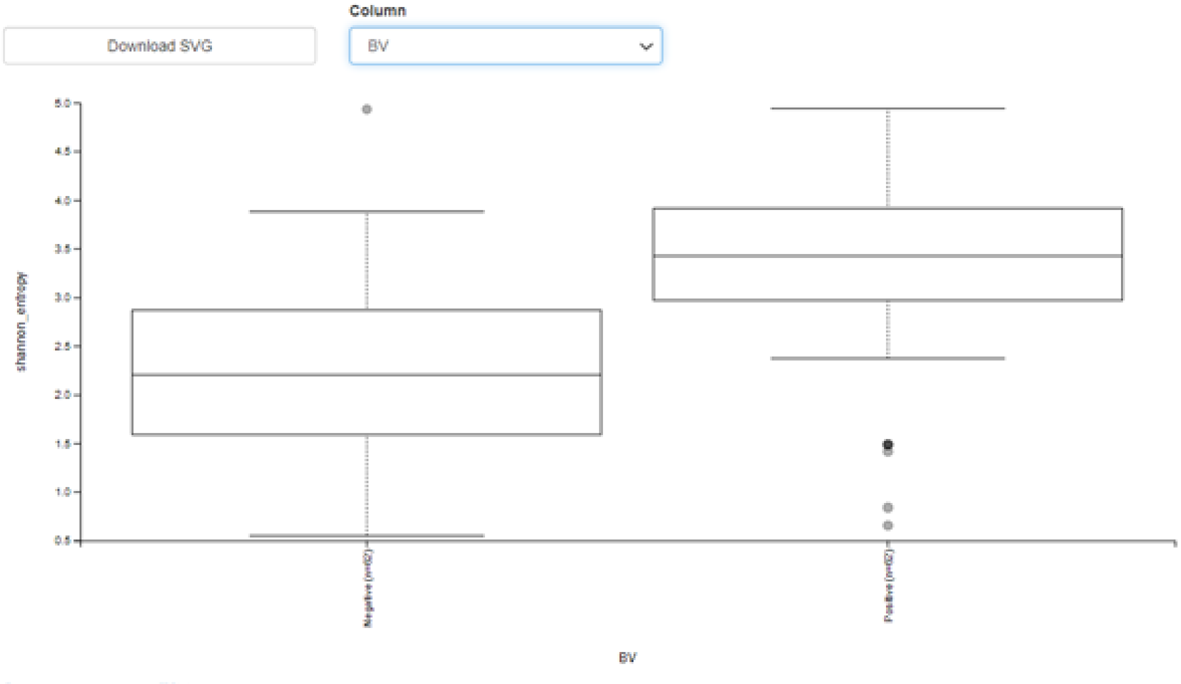
Shannon index is a measure of entropy, that is, the amount of information in a system where the unit is bits of information if the logarithms are base 2. The diagrams below illustrate the Shannon diversity index for each parameter.

Bacteria Vaginosis shannon index

Shannon index for inflammation

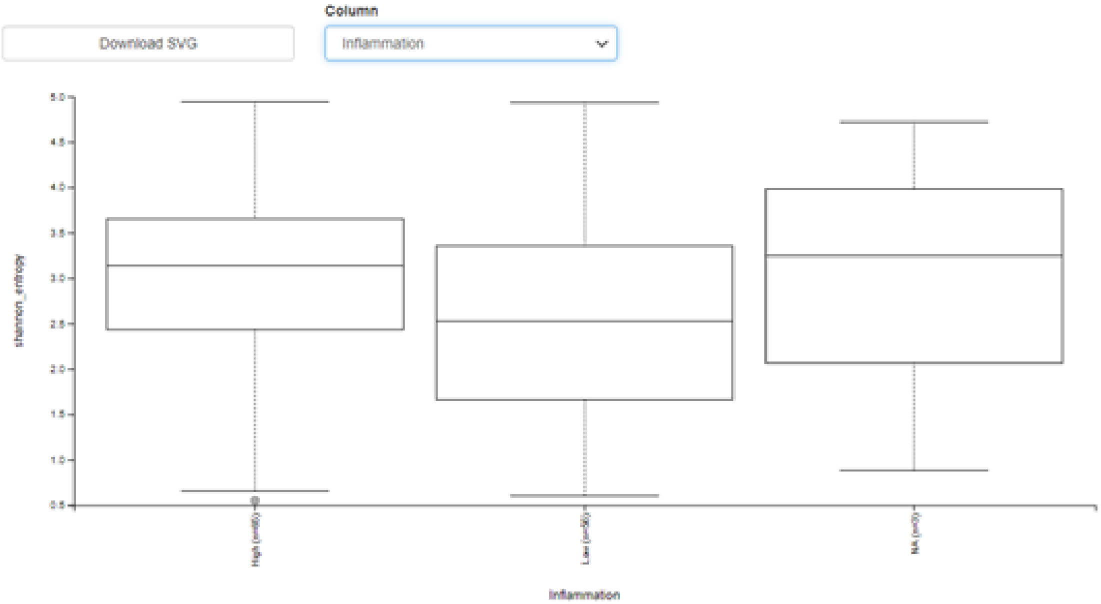
Body Mass Index

Age



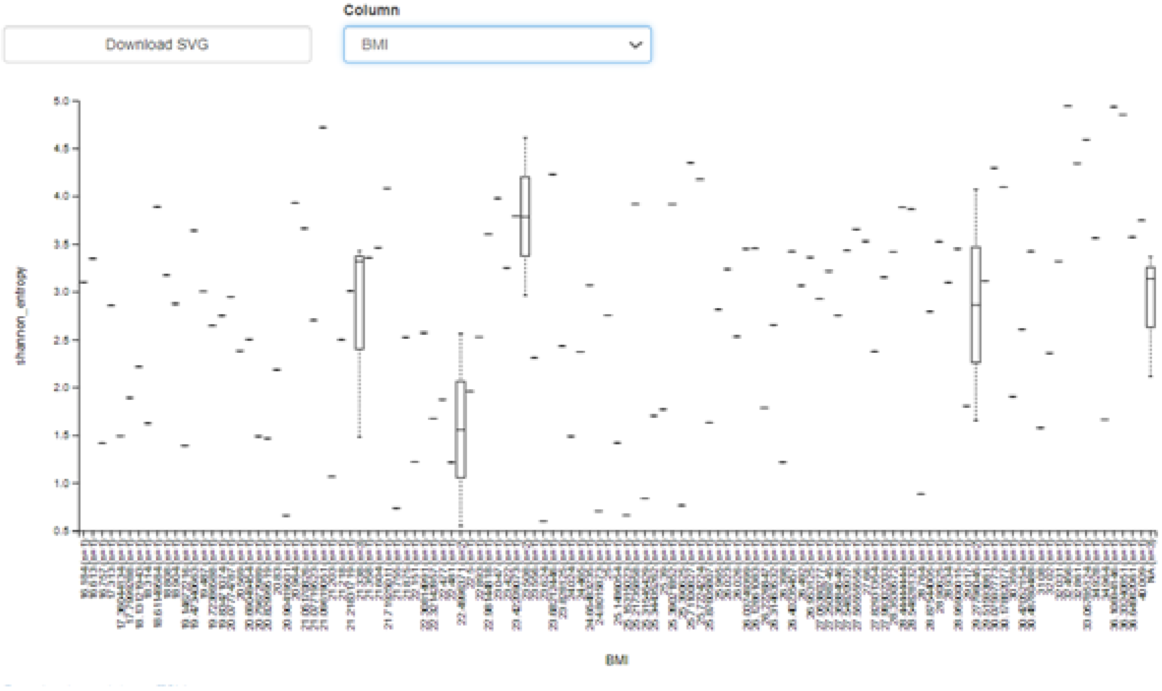
Column: BV

Figure 11: BV_shannon



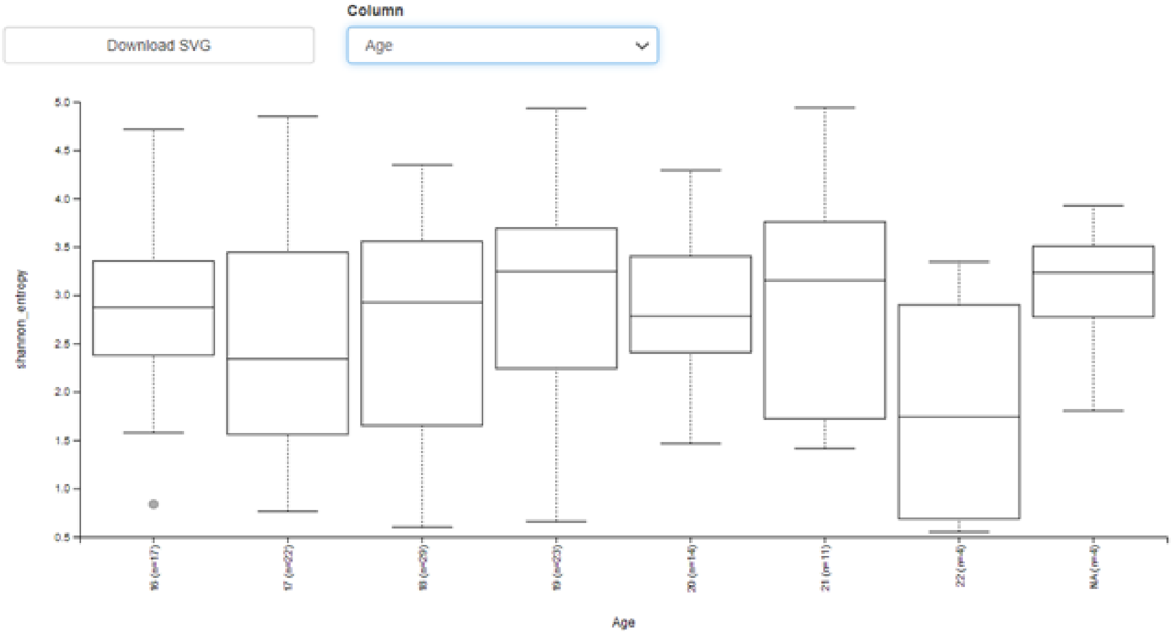
Column: Inflammation

Figure 12: Inflammation_Shannon



Column: BMI

Figure 13: BMI_Shannon



Column: Age

Figure 14: Age_Shannon