Alpha and Beta Diversity

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Alpha and Beta Diversity Analysis Tutorial

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This tutorial will cover mctoolsr and some commonly used alpha and beta diversity statistics and plotting

NOTE: You can do many of the same things with the phyloseq package. We use mctoolsR mainly because it was designed by a former student in the Fierer Lab (Jon Leff) and our dada2 pipeline is set up to export into the format required.

Setup

Note the code below uses the example dataset from mctoolsR (https://github.com/leffj/mctoolsr). I will note where you can change the inputs and code to use your own data instead

```r
#R packages required for this tutorial
library(mctoolsr) # For inputting, rarefying, filtering, taxonomic analyses
```

```
## You're using mctoolsr (v.0.1.1.9). Direct inquiries to:
## 'https://github.com/leffj/mctoolsr'
```

```r
library(plyr) # For hulls
library(tidyverse) # For data manipulation and plotting
```

```
## -- Attaching core tidyverse packages ------------------------ tidyverse 2.0.0 --
## v dplyr     1.1.3     v readr     2.1.4
## v forcats   1.0.0     v stringr   1.5.0
## v ggplot2   3.4.3     v tibble    3.2.1
## v lubridate 1.9.2     v tidyr     1.3.0
## v purrr     1.0.2
```

```
## -- Conflicts ------------------------------------------ tidyverse_conflicts() --
## x dplyr::arrange()   masks plyr::arrange()
## x purrr::compact()   masks plyr::compact()
## x dplyr::count()     masks plyr::count()
## x dplyr::desc()      masks plyr::desc()
## x dplyr::failwith()  masks plyr::failwith()
## x dplyr::filter()    masks stats::filter()
## x dplyr::id()        masks plyr::id()
## x dplyr::lag()       masks stats::lag()
## x dplyr::mutate()    masks plyr::mutate()
## x dplyr::rename()    masks plyr::rename()
## x dplyr::summarise() masks plyr::summarise()
## x dplyr::summarize() masks plyr::summarize()
## i Use the conflicted package (<http://conflicted.r-lib.org/>) to force all conflicts to become error
```

```r
library(RColorBrewer) # For color palettes
library(vegan) # For alpha and beta diversity analyses
```

```
## Loading required package: permute
## Loading required package: lattice
## This is vegan 2.6-4
```

```r
library(indicspecies) # For indicator species
library(car) # For Levene Test and anova
```

```
## Loading required package: carData
##
## Attaching package: 'car'
##
## The following object is masked from 'package:dplyr':
##
##     recode
##
## The following object is masked from 'package:purrr':
##
##     some
```

```r
library(PMCMRplus) # For Nemenyi posthoc test
library(ggh4x) # For nested facets
library(emmeans) # For pairwise comparisons
library(multcomp) # For automated significant difference letters
```

```
## Loading required package: mvtnorm
## Loading required package: survival
## Loading required package: TH.data
## Loading required package: MASS
##
## Attaching package: 'MASS'
##
## The following object is masked from 'package:dplyr':
##
##     select
##
##
## Attaching package: 'TH.data'
##
## The following object is masked from 'package:MASS':
##
##     geyser
```

```r
library(zCompositions) # CLR
```

```
## Loading required package: NADA
##
## Attaching package: 'NADA'
##
## The following object is masked from 'package:stats':
##
##     cor
##
## Loading required package: truncnorm
```

```r
library(compositions) # Aitchison
```

```
## Welcome to compositions, a package for compositional data analysis.
## Find an intro with "? compositions"
##
##
## Attaching package: 'compositions'
##
## The following object is masked from 'package:NADA':
##
##     cor
##
## The following objects are masked from 'package:stats':
##
##     anova, cor, cov, dist, var
##
## The following object is masked from 'package:graphics':
##
##     segments
##
## The following objects are masked from 'package:base':
##
##     %*%, norm, scale, scale.default
```

```r
library(reshape2) # For melting
```

```
##
## Attaching package: 'reshape2'
##
## The following object is masked from 'package:tidyr':
##
##     smiths
```

```r
library(cowplot) # For plotting
```

```
##
## Attaching package: 'cowplot'
##
## The following object is masked from 'package:lubridate':
##
##     stamp
```

```r
##If you haven't already installed these packages before, you will need to install them with:
#install.packages()
```

```
##For Installing mctoolsR the first time, use:
#install.packages("devtools")
#devtools::install_github("leffj/mctoolsr")

# Custom functions
source("cliffplot_taxa_bars.R")
source("plot_multipatt_asv.R")
```

Loading Data

This loads the mctoolsr example dataset.

```
#Loading ASV Table
tax_table_fp = system.file('extdata', 'fruits_veggies_taxa_table_wTax.biom',
                           package = 'mctoolsr')

#Loading mapping file
map_fp = system.file('extdata', 'fruits_veggies_metadata.txt',
                     package = 'mctoolsr')

# To load YOUR OWN data, replace everything after the "=" with: 'PATH/TO/YOUR_ASV_TABLE_FILE.txt' <- ou
# If your files are in your working directly, you do not need to specify the path

# Combine the ASV table and mapping file into an mctoolsR object
input = load_taxa_table(tax_table_fp, map_fp)
```

```
## 32 samples loaded
```

```
#If this has worked correctly, you should get a note saying how many samples have been loaded. For the
```

#Understanding the mctoolsR dataframe ## The mctoolsR object is a list with 3 dataframes that can be accessed with $. The dataframes are a sequence table, a mapping file, and a taxonomic file.

```
# Examine the input dataframes.
head(input$data_loaded) # Rows are OTUs, columns are samples
```

```
##        ProA12 ProA13 ProA14 ProA15 ProA16 ProA33 ProA34 ProA35 ProA36 ProA37
## OTU_2       0      0      0      0      0      0      0      0      2      0
## OTU_3       0      0      0      0      0      1      0      0      0      0
## OTU_12      0      0      0      0      0      0      0      0      0      0
## OTU_13      0      1      0      0      0      0      0      0      0      0
## OTU_20      2      0      0      0      0      0      0      0      0      0
## OTU_25      0      0      0      0      1      0      0      0      0      0
##        ProA58 ProA65 ProA66 ProB10 ProB12 ProB33 ProB34 ProB35 ProB36 ProB39
## OTU_2       0      0      0      0      0      0      0      0      0      0
## OTU_3       0      0      0      0      0      0      0      0      0      0
## OTU_12      0      0      0      0      0      0      0      0      0      0
## OTU_13      0      0      0      0      0      0      0      0      0      0
## OTU_20      0      0      0      0      0      0      0      0      0      0
## OTU_25      0      0      0      0      0      0      0      0      0      0
##        ProB40 ProB57 ProB58 ProB60 ProB67 ProB70 ProB71 ProB9 ProC36 ProC40
## OTU_2       0      0      0      0      0      0      0     0      0      0
## OTU_3       0      0      0      0      0      0      0     0      0      0
## OTU_12      0      1      0      0      0      0      0     0      0      0
## OTU_13      0      0      0      0      0      0      0     0      0      0
## OTU_20      0      0      0      0      0      0      0     2      0      0
## OTU_25      0      0      0      0      0      0      0     0      0      0
##        ProC65 ProC66
## OTU_2       0      0
## OTU_3       0      0
## OTU_12      0      0
## OTU_13      0      0
## OTU_20      0      0
## OTU_25      0      0
```

```
head(input$taxonomy_loaded) # Rows are OTUs (now ASVs), columns are different taxonomic levels
```

```
##          taxonomy1         taxonomy2              taxonomy3
## OTU_2  k__Bacteria p__Actinobacteria      c__Actinobacteria
## OTU_3  k__Bacteria p__Proteobacteria c__Gammaproteobacteria
## OTU_12 k__Bacteria     p__Firmicutes             c__Bacilli
## OTU_13  Unassigned      unclassified           unclassified
## OTU_20 k__Bacteria p__Proteobacteria c__Gammaproteobacteria
## OTU_25  Unassigned      unclassified           unclassified
##                  taxonomy4             taxonomy5        taxonomy6    taxonomy7
## OTU_2    o__Actinomycetales f__Pseudonocardiaceae g__Amycolatopsis          s__
## OTU_3  o__Enterobacteriales f__Enterobacteriaceae              g__          s__
## OTU_12        o__Bacillales        f__Bacillaceae      g__Bacillus          s__
## OTU_13         unclassified          unclassified     unclassified unclassified
## OTU_20     o__Aeromonadales     f__Aeromonadaceae              g__          s__
## OTU_25         unclassified          unclassified     unclassified unclassified
```

```
head(input$map_loaded) # Rows are samples, columns are variables
```

```
##         Sample_type    Farm_type            Sample_Farming
## ProA12    Mushrooms Conventional    Mushrooms_Conventional
## ProA13    Mushrooms      Organic         Mushrooms_Organic
## ProA14    Mushrooms      Organic         Mushrooms_Organic
## ProA15    Mushrooms      Organic         Mushrooms_Organic
## ProA16    Mushrooms      Organic         Mushrooms_Organic
## ProA33 Strawberries Conventional Strawberries_Conventional
```

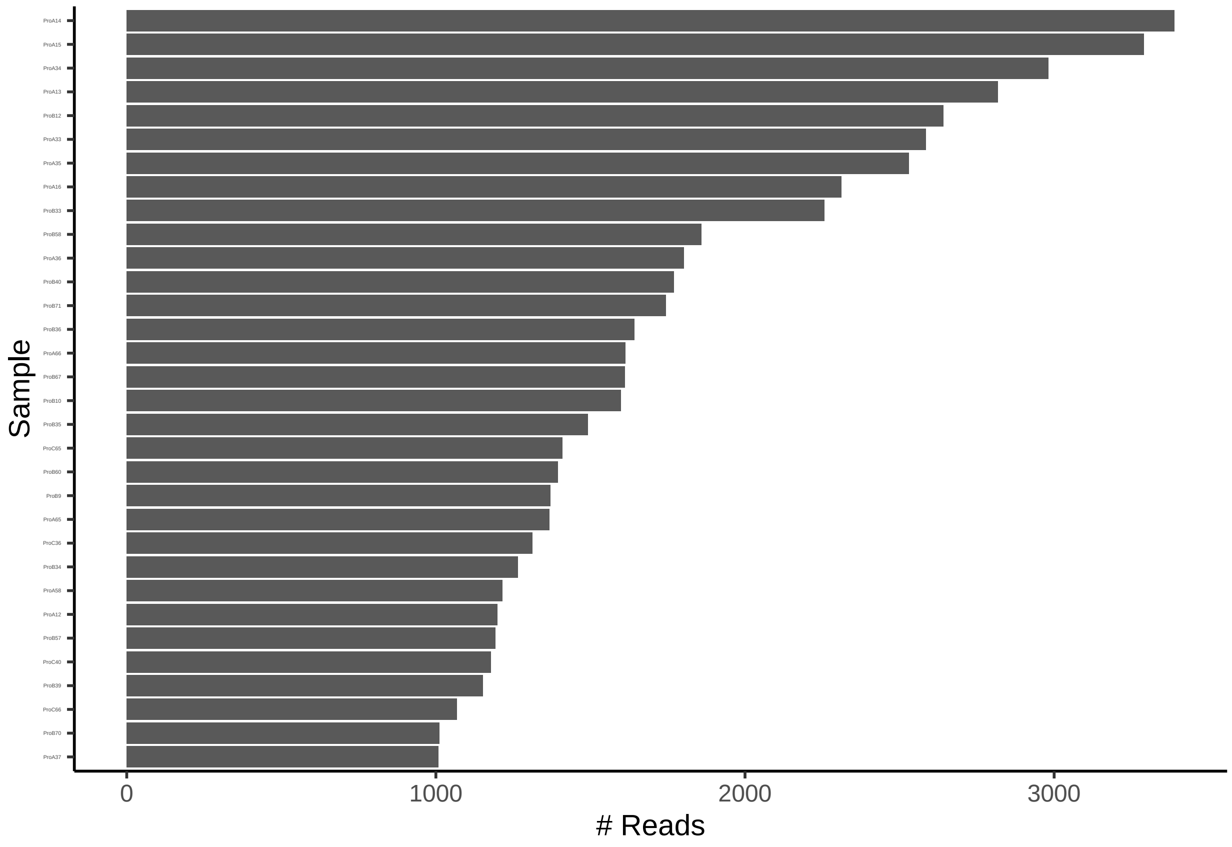
Initial data examinination - reads per sample

```
# One of the first things we want to do is see how many sequences per sample there are
# This is done by getting the column sums of the sequence table, and we'll sort it too.
sort(colSums(input$data_loaded))
```

```
## ProA37 ProB70 ProC66 ProB39 ProC40 ProB57 ProA12 ProA58 ProB34 ProC36 ProA65
##   1009   1011   1068   1152   1179   1192   1199   1216   1265   1313   1367
##  ProB9 ProB60 ProC65 ProB35 ProB10 ProB67 ProA66 ProB36 ProB71 ProB40 ProA36
##   1371   1395   1409   1492   1599   1611   1614   1642   1745   1771   1802
## ProB58 ProB33 ProA16 ProA35 ProA33 ProB12 ProA13 ProA34 ProA15 ProA14
##   1860   2257   2312   2530   2585   2642   2819   2982   3291   3390
```

```
# We could also save this as an object and plot it
seqcounts <- as.data.frame(sort(colSums(input$data_loaded)))

# This puts the sample names as row names and the sequence counts column is titled "sort(colSums(input$
```

```r
# We'll use the pipeline %>% which is very useful for dataframe management with the dplyr package
# We'll rename the column and make a new column all at once
seqcounts <- as.data.frame(sort(colSums(input$data_loaded))) %>%
  rename("seqs" = "sort(colSums(input$data_loaded))") %>%
  rownames_to_column(var = "sampleID")

# Now we have a better dataframe with two columns, seqs and sampleID which we can plot
ggplot(seqcounts, aes(reorder(sampleID, seqs, mean), seqs)) + # Dataframe and variables
  geom_bar(stat = "identity") + # Type of graph
  labs(y = "# Reads", x = "Sample") + # Axes labels
  coord_flip() + # Flip axes
  theme_classic() + # Plot style. I also use theme_bw() a lot.
  theme(axis.text.y = element_text(size = 2)) # Tweak things about the text and legend in theme()
```



Filtering data

We often filter out anything not assigned to a phylum and any control samples. You may also want to do analyses on only a subset of your data, or remove contaminant ASVs that are found in the PCR or extraction blanks. For 16S, at the least filter out mitochondrial, chloroplast, eukaryote DNA, and things unassigned at phylum level. Depending on your samples and analysis goals, you should also consider filtering out extremely rare amplicon sequence variants. Here we will demonstrate removal of what are called "singletons" and "doubletons", which are individual amplicon sequence variants that only appear once or twice, respectively, in the whole dataset. This is generally recommended.

```
# Copy the dataset before doing any filtering
input_filt <- input

# For all filtering steps, taxa removed will be output, make note of this for methods section. Also som

# This filters out any ASVs from input dataframe ("input_filt") not assigned to a phylum (at taxonomy l
input_filt <- filter_taxa_from_input(input_filt,
                                     at_spec_level = 2,
                                     taxa_to_remove = "unclassified") # 978
```

```
## 978 taxa removed
```

```
# New versions call it NA
#input_filt <- filter_taxa_from_input(input_filt, at_spec_level = 2, taxa_to_remove = "NA")

# Filtering out mitochondria sequences from the filtered input dataset.
input_filt <- filter_taxa_from_input(input_filt,
                                     taxa_to_remove = "f__mitochondria") # 14
```

```
## 14 taxa removed
```

```
# New versions call it Mitochondria
#input_filt <- filter_taxa_from_input(input_filt, taxa_to_remove = "Mitochondria")

# Filtering out chloroplast sequences
input_filt <- filter_taxa_from_input(input_filt,
                                     taxa_to_remove = "c__Chloroplast") # 1
```

```
## 1 taxa removed
```

```
# New versions call it Chloroplast
#input_filt <- filter_taxa_from_input(input_filt, taxa_to_remove = "Chloroplast")

# Filtering out Eukaryotes
#input_filt <- filter_taxa_from_input(input_filt, taxa_to_remove = "Eukaryota") # 0

# If you want to filter out multiple groups at the same time, you can also do that like this:
# input_filt <- filter_taxa_from_input(input_filt,
#                                      taxa_to_remove = c("Chloroplast",
#                                                         "Mitochondria",
#                                                         "Eukaryota"))
# However, it's interesting to do it individually to see how much of each DNA category there was!
```

```r
# Filtering out singletons and doubletons
# First we'll save the ASV IDs of ASVs with less than 3 total counts
singdoub <- data.frame("count" = rowSums(input_filt$data_loaded)) %>%
  filter(count < 3) %>%
  mutate(ASV = rownames(.))

# Now we'll provide that list of ASV IDs to the taxa_IDs_to_remove argument
input_filt <- filter_taxa_from_input(input_filt,
                                     taxa_IDs_to_remove = singdoub$ASV)
```

```
## 2091 taxa removed
```

```r
# NOTE: Each line you run updates the input_filt dataset, so be careful, if something goes wrong go bac

# NOTE: Databases update and sometimes change how Chloroplast, mitochondria, Eukaryotes are named. If y

# NOTE: You can also use taxa_to_keep, specify the taxonomic level with at_spec_level, remove or keep i

# In addition to taxonomic filtering, you can filter out samples. For example, let's filter out lettuce
input_filt <- filter_data(input_filt, # provide input_filt again. careful!
                          filter_cat = "Sample_type",
                          filter_vals = "Lettuce") # 28 samples remaining (removed 4)
```

```
## 28 samples remaining
```

```r
# Note: normally after you've filtered and rarefied it's recommended to save the dataset as a .rds file

# Lets save the filtered but unrarefied dataset.
saveRDS(input_filt, file = "input_filt.rds")

# You can read it in like this:
input_filt <- readRDS("input_filt.rds")
```

Rarefying data

```r
# Moving on, let's rarefy the data at the lowest count per sample. Sometimes you may want to drop sampl

# Let's recheck the reads per sample now that we've filtered out a lot of crap.
sort(colSums(input_filt$data_loaded))
```

```
## ProB70 ProA37 ProC66 ProA12 ProB39 ProC40 ProC36  ProB9 ProB34 ProA65 ProC65
##    949    964   1020   1061   1113   1114   1118   1194   1208   1302   1355
## ProB10 ProB35 ProB71 ProB67 ProA66 ProB36 ProA36 ProB40 ProA16 ProB33 ProB12
##   1383   1458   1463   1491   1514   1585   1696   1743   2056   2111   2165
## ProA35 ProA13 ProA33 ProA34 ProA15 ProA14
##   2378   2390   2428   2790   2828   3000
```

```r
# Rarefy at 949 seqs/sample
set.seed(600) # Set a seed to make this reproducible, although you can also just write to disk and relo
input_filt_rar = single_rarefy(input_filt, 949) # This makes a new mctoolsR dataset called input_filt_r
```

```
## 28 samples remaining
```

```r
# The number of samples remaining is stated. Any samples below the threshold get dropped.
# Check seq counts in new dataset
colSums(input_filt_rar$data_loaded) # Good - all 949! It worked.
```

```
## ProA12 ProA13 ProA14 ProA15 ProA16 ProA33 ProA34 ProA35 ProA36 ProA37 ProA65
##    949    949    949    949    949    949    949    949    949    949    949
## ProA66 ProB10 ProB12 ProB33 ProB34 ProB35 ProB36 ProB39 ProB40 ProB67 ProB70
##    949    949    949    949    949    949    949    949    949    949    949
## ProB71  ProB9 ProC36 ProC40 ProC65 ProC66
##    949    949    949    949    949    949
```

```r
# Save the file. You can reload it with readRDS().
saveRDS(input_filt_rar, file = "input_filt_rar.rds")
input_filt_rar <- readRDS("input_filt_rar.rds")
```

#ALPHA DIVERSITY ##ASV richness and Shannon diversity.

```r
# Let's analyze and graph the number of OTUs (now ASVs) in our sample types

# First let's get the number of ASVs (richness) per sample and add it to the mapping file
input_filt_rar$map_loaded$rich <- specnumber(input_filt_rar$data_loaded,
                                             MARGIN = 2)

# Now let's get the Shannon diversity index and add it to the mapping file
# Note: Shannon diversity takes into account the ASV richness, as well as evenness
input_filt_rar$map_loaded$shannon <- diversity(input_filt_rar$data_loaded,
                                               index = "shannon",
                                               MARGIN = 2)

# Note: since the data_loaded file has ASVs as rows, MARGIN must be set to 2.
```

Two categories: t-test (parametric) or Wilcoxon Test (non-parametric)

```r
# Two categories example
leveneTest(input_filt_rar$map_loaded$rich ~ input_filt_rar$map_loaded$Farm_type)
```

```
## Warning in leveneTest.default(y = y, group = group, ...): group coerced to
## factor.

## Levene's Test for Homogeneity of Variance (center = median)
##       Df F value Pr(>F)
## group  1  0.0522 0.8211
##       26
```

```r
# Variance homogeneous (p > 0.05)

shapiro.test(input_filt_rar$map_loaded$rich)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  input_filt_rar$map_loaded$rich
## W = 0.97153, p-value = 0.6221
```

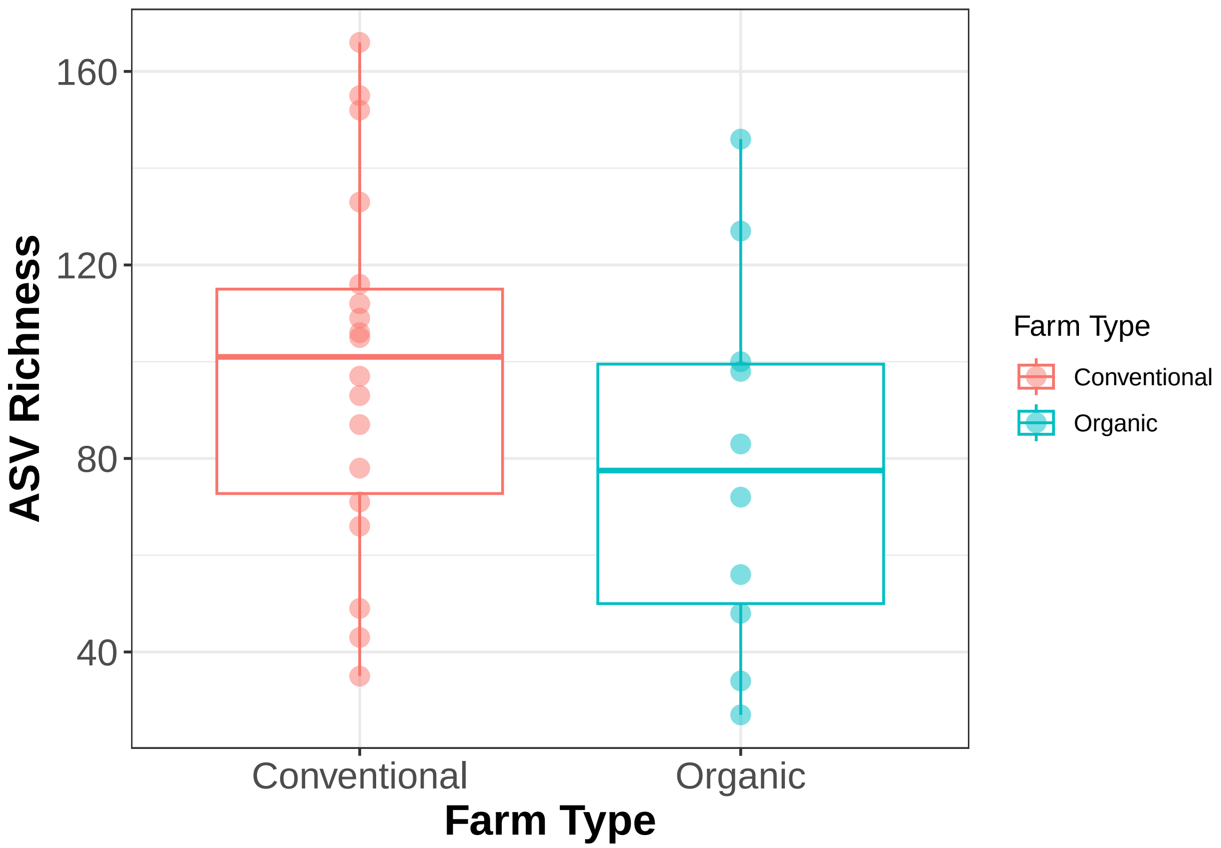
```r
# Richness normally distributed (p > 0.05)

t.test(input_filt_rar$map_loaded$rich ~ input_filt_rar$map_loaded$Farm_type)
```

```
##
##  Welch Two Sample t-test
##
## data:  input_filt_rar$map_loaded$rich by input_filt_rar$map_loaded$Farm_type
## t = 1.2678, df = 18.164, p-value = 0.2209
## alternative hypothesis: true difference in means between group Conventional and group Organic is not
## 95 percent confidence interval:
##  -12.72671  51.52671
## sample estimates:
## mean in group Conventional      mean in group Organic
##                       98.5                       79.1
```

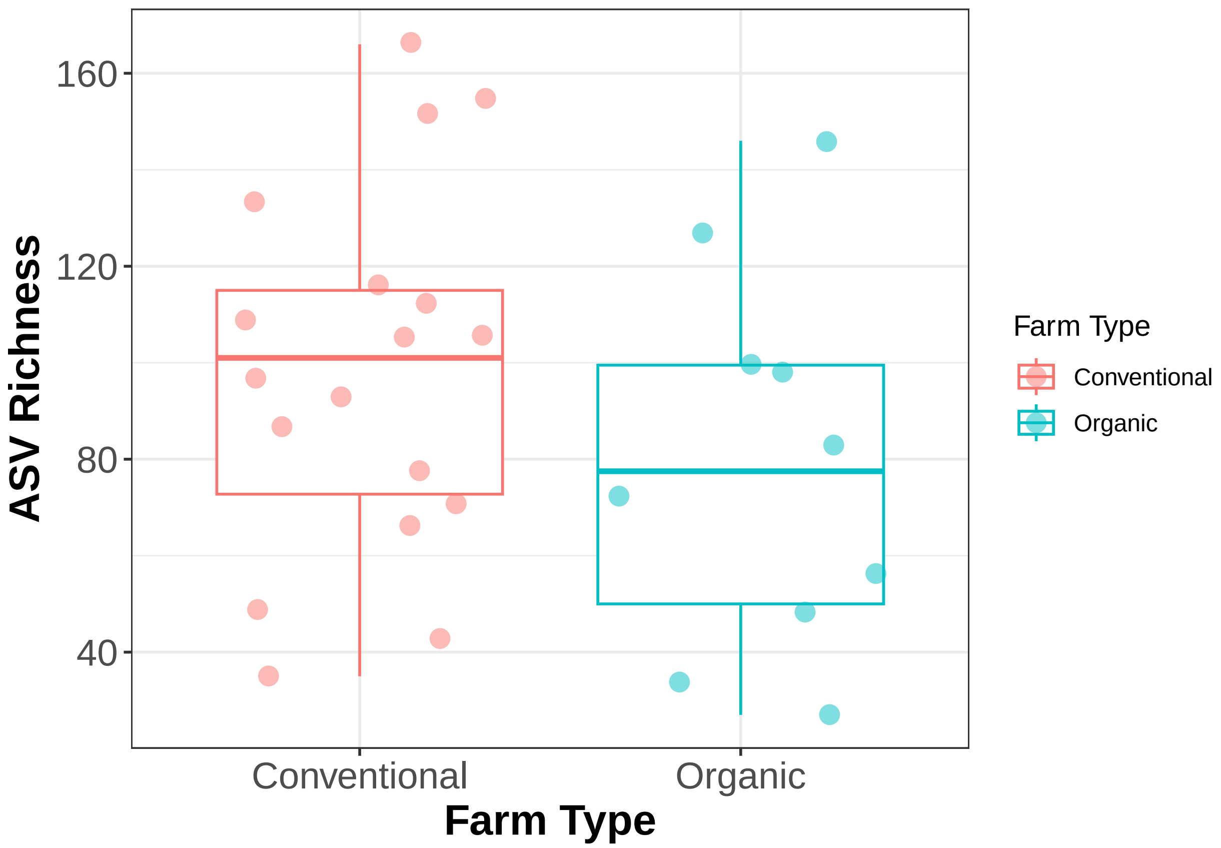
```
# No significant difference in richness among the two categories

# If Levene's Test or Shapiro-Wilk Test p < 0.05, use Wilcoxon Test
wilcox.test(input_filt_rar$map_loaded$rich ~ input_filt_rar$map_loaded$Farm_type)
```

```
##
##  Wilcoxon rank sum exact test
##
## data:  input_filt_rar$map_loaded$rich by input_filt_rar$map_loaded$Farm_type
## W = 117, p-value = 0.2079
## alternative hypothesis: true location shift is not equal to 0
```

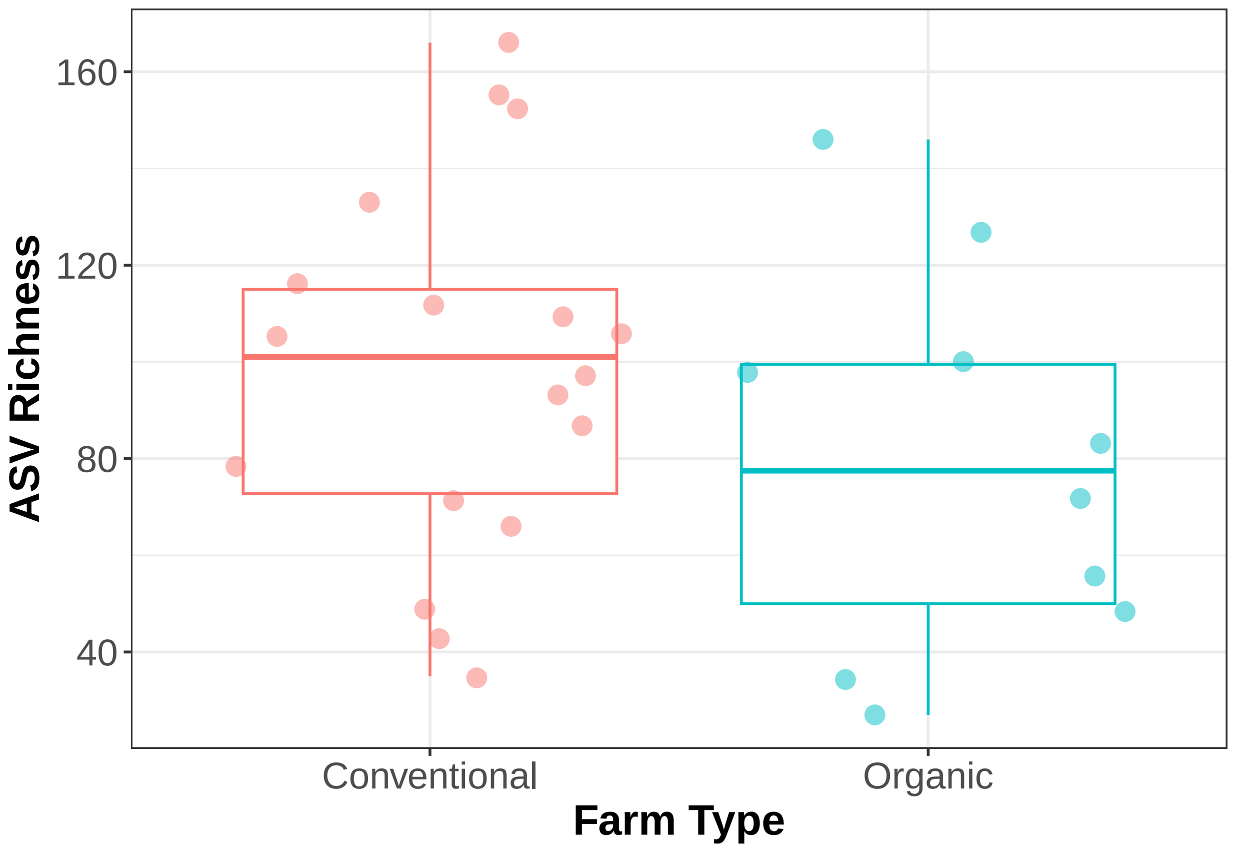
```
# Same result

# Box and whisker plot with points
ggplot(input_filt_rar$map_loaded, aes(Farm_type, rich, colour = Farm_type)) +
  geom_boxplot(outlier.shape = NA) +
  geom_point(size = 3, alpha = 0.5) +
  labs(x = "Farm Type", y = "ASV Richness", colour = "Farm Type") +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14))
```



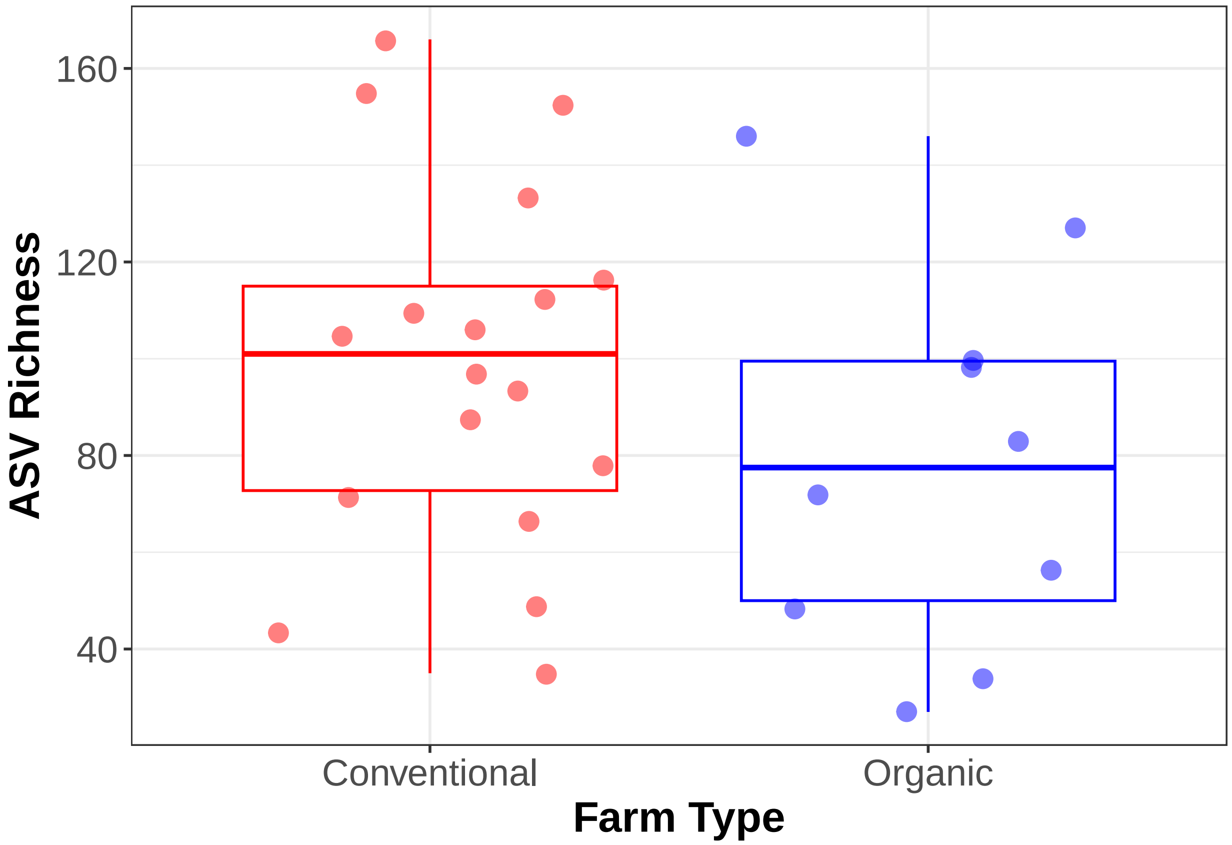
```r
# Box and whisker plot with jittered points (geom_jitter instead of geom_point)
ggplot(input_filt_rar$map_loaded, aes(Farm_type, rich, colour = Farm_type)) +
  geom_boxplot(outlier.shape = NA) +
  geom_jitter(size = 3, alpha = 0.5) +
  labs(x = "Farm Type", y = "ASV Richness", colour = "Farm Type") +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14))
```



```r
# Don't need legend because each category is already clearly labeled
ggplot(input_filt_rar$map_loaded, aes(Farm_type, rich, colour = Farm_type)) +
  geom_boxplot(outlier.shape = NA) +
  geom_jitter(size = 3, alpha = 0.5) +
  labs(x = "Farm Type", y = "ASV Richness", colour = "Farm Type") +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14),
        legend.position = "none")
```



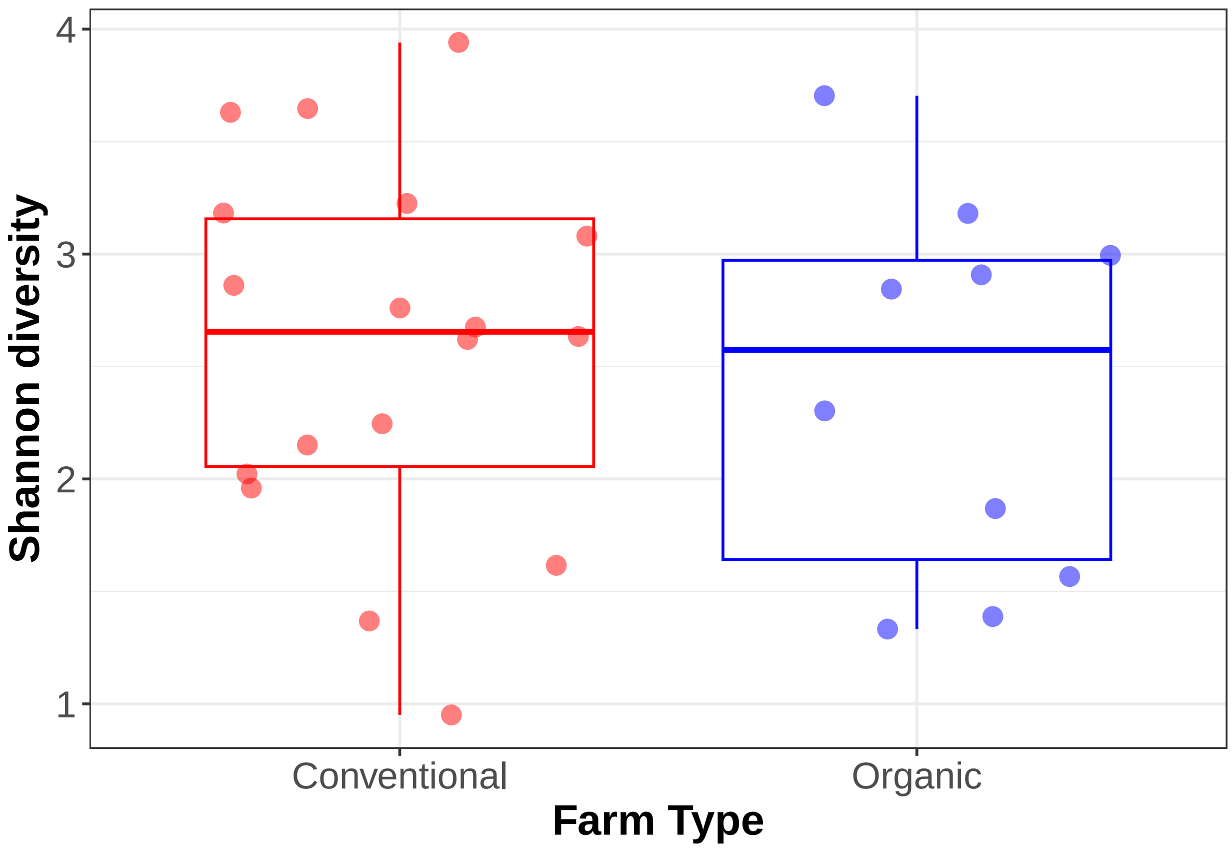
```r
# Change colors to be colorblind friendly (e.g., red and blue is better than red and green)
ggplot(input_filt_rar$map_loaded, aes(Farm_type, rich, colour = Farm_type)) +
  geom_boxplot(outlier.shape = NA) +
  geom_jitter(size = 3, alpha = 0.5) +
  labs(x = "Farm Type", y = "ASV Richness", colour = "Farm Type") +
  scale_colour_manual(values = c("red", "blue")) +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14),
        legend.position = "none")
```



```r
# Save plot. This specifies a size and makes it reproducible. pdf default dimensions are in inches. cou
pdf(file = "FarmType.pdf", width = 4, height = 4)
ggplot(input_filt_rar$map_loaded, aes(Farm_type, rich, colour = Farm_type)) +
  geom_boxplot(outlier.shape = NA) +
  geom_jitter(size = 3, alpha = 0.5) +
  labs(x = "Farm Type", y = "ASV Richness", colour = "Farm Type") +
  scale_colour_manual(values = c("red", "blue")) +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14),
        legend.position = "none")
dev.off()
```

```
## pdf
##   2
```

```r
# Now let's plot Shannon diversity. I cut and pasted the above plot and changed the y input and axis la
ggplot(input_filt_rar$map_loaded, aes(Farm_type, shannon, colour = Farm_type)) +
  geom_boxplot(outlier.shape = NA) +
  geom_jitter(size = 3, alpha = 0.5) +
  labs(x = "Farm Type", y = "Shannon diversity", colour = "Farm Type") +
  scale_colour_manual(values = c("red", "blue")) +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14),
        legend.position = "none")
```



Three + categores: ANOVA (parametric) or Kruskal-Wallis Test (non-parametric)

```
# Three category example
leveneTest(input_filt_rar$map_loaded$rich ~ input_filt_rar$map_loaded$Sample_type)
```

```
## Warning in leveneTest.default(y = y, group = group, ...): group coerced to
## factor.
```

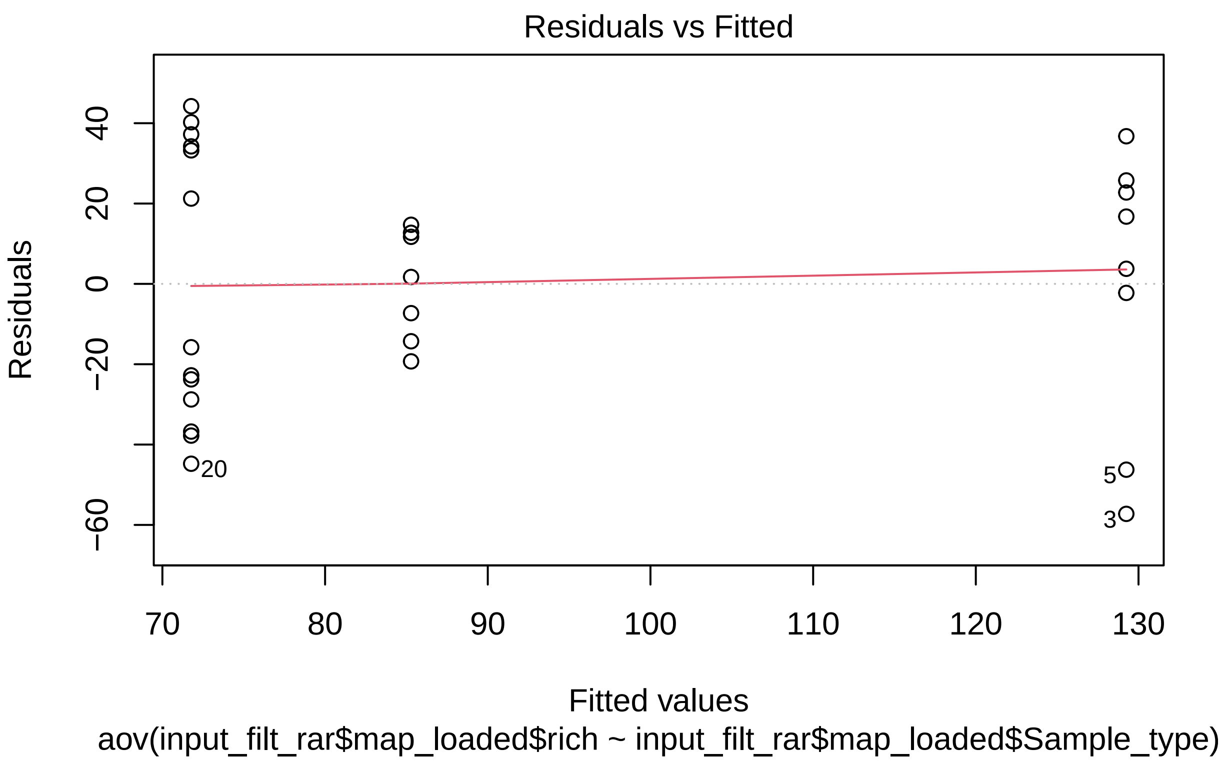
```
## Levene's Test for Homogeneity of Variance (center = median)
##       Df F value Pr(>F)
## group  2  2.3697 0.1142
##       25
```

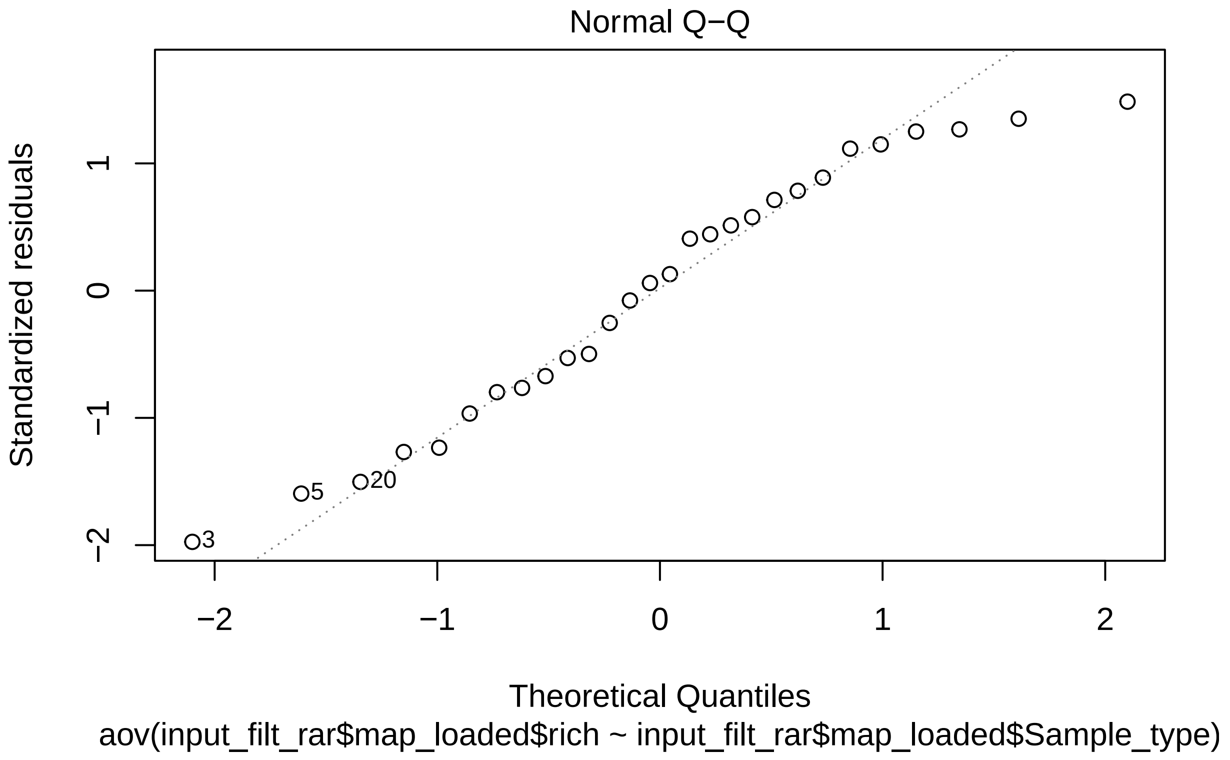
```
# Variance homogeneous (p > 0.05)

m <- aov(input_filt_rar$map_loaded$rich ~ input_filt_rar$map_loaded$Sample_type)
shapiro.test(m$residuals)
```

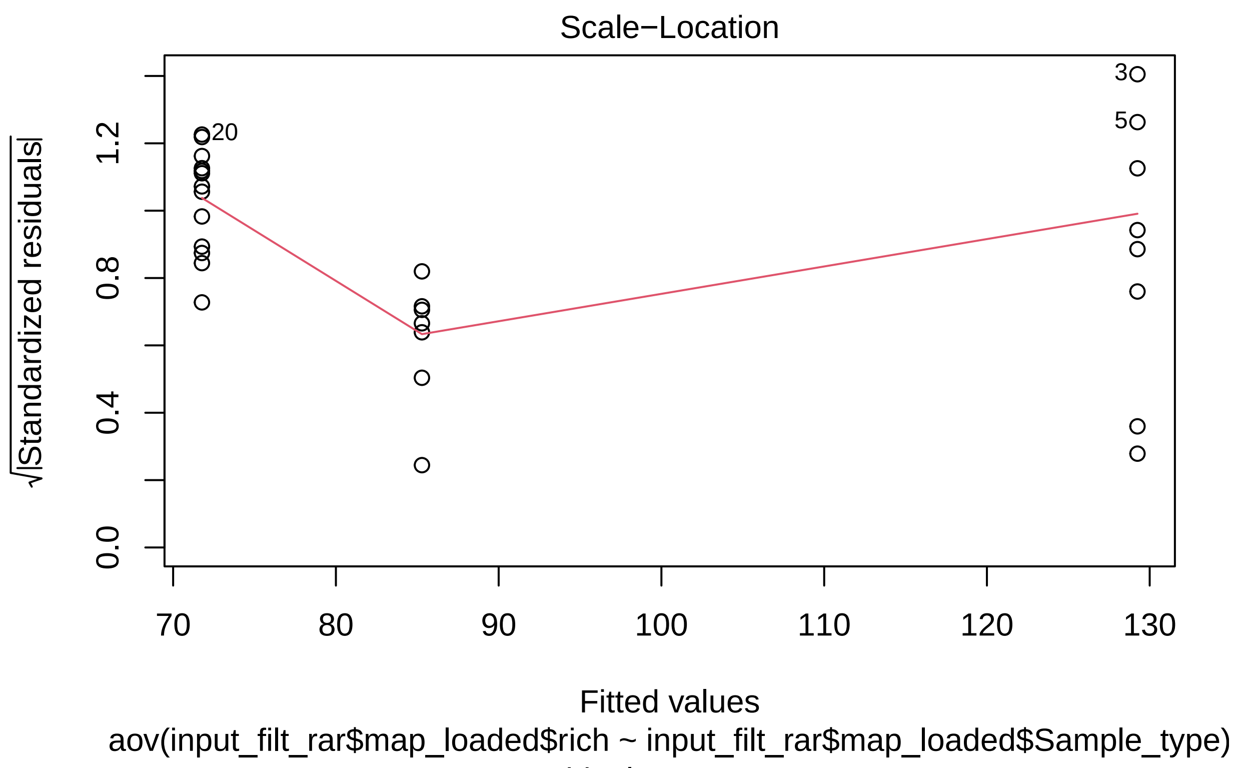
```
##
##  Shapiro-Wilk normality test
##
## data:  m$residuals
## W = 0.95207, p-value = 0.2232
```

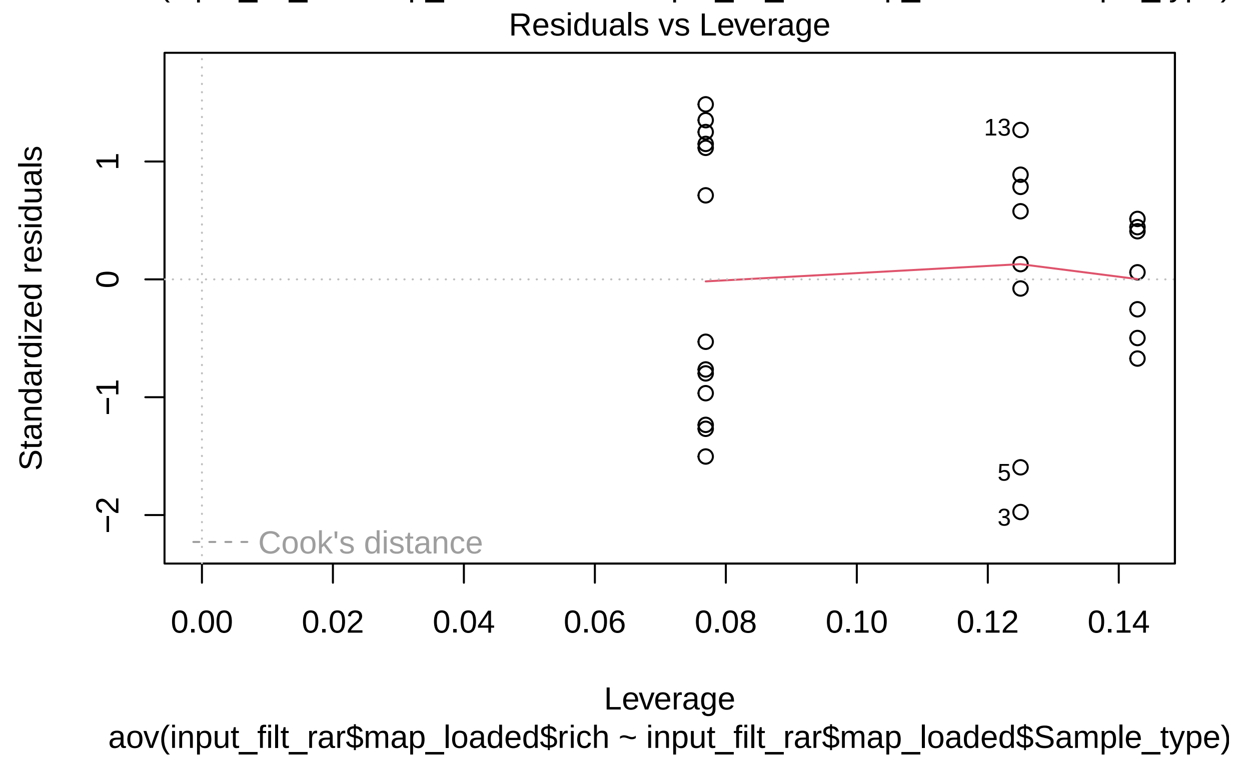
```
# Residuals normally distributed (p > 0.05)
# Note: Here we test the assumption that the residuals (not the data itself) are normally distributed
```

```r
# Other diagnostics - learn more here (https://data.library.virginia.edu/diagnostic-plots/)
plot(m) # Click in the console and hit Return to see each diagnostic plot
```









```
summary(m)
```

```
##                                       Df Sum Sq Mean Sq F value  Pr(>F)
## input_filt_rar$map_loaded$Sample_type  2  16732    8366   8.705 0.00135 **
## Residuals                             25  24025     961
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```r
# Significant effect. Run posthoc test to see which groups are different from each other.

TukeyHSD(m)
```

```
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = input_filt_rar$map_loaded$rich ~ input_filt_rar$map_loaded$Sample_type)
##
## $`input_filt_rar$map_loaded$Sample_type`
##                              diff       lwr       upr     p adj
## Spinach-Mushrooms      -43.96429 -83.92742  -4.00115 0.0290426
## Strawberries-Mushrooms -57.48077 -92.17849 -22.78305 0.0010087
## Strawberries-Spinach   -13.51648 -49.71596  22.68299 0.6267806
```

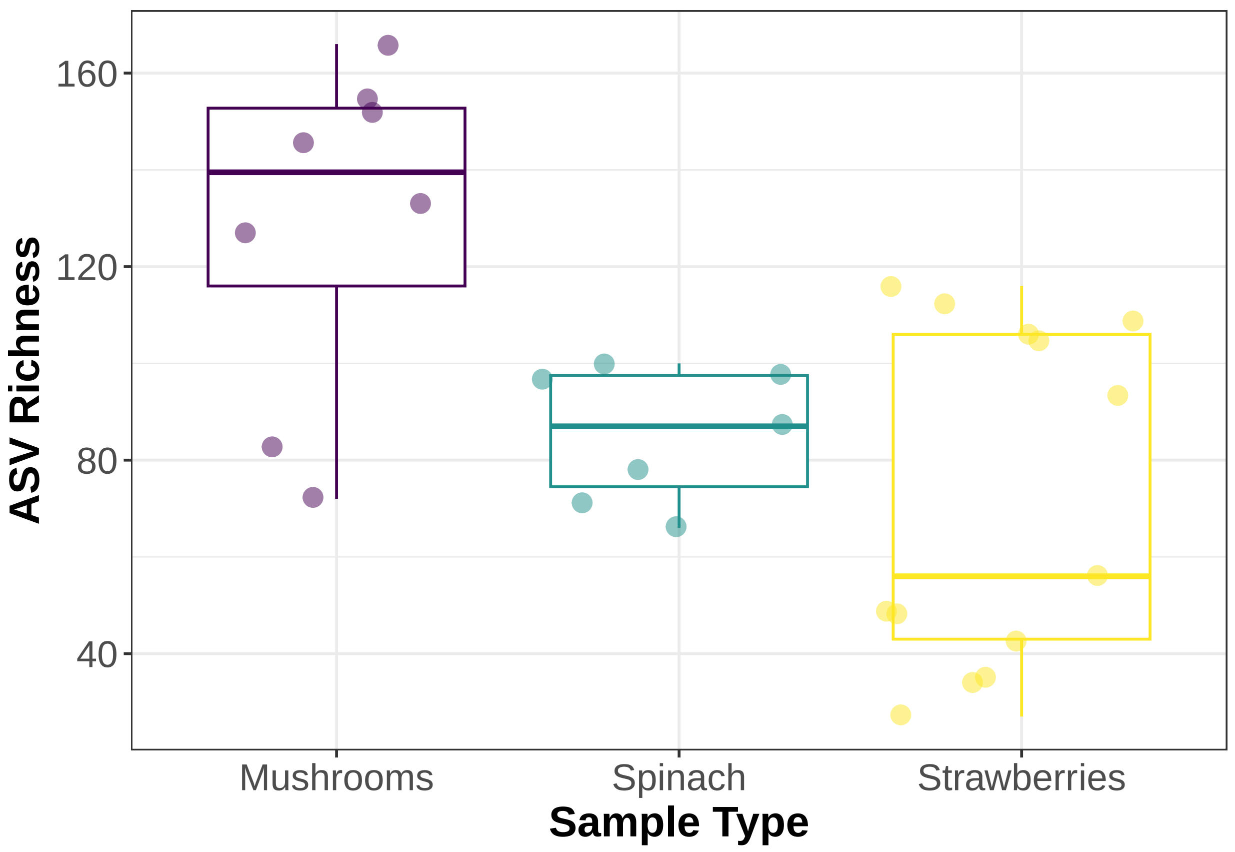
```r
# Mushrooms different from spinach and strawberries. No diff between spinach and strawberries

# If Levene's Test or Shapiro-Wilk Test p < 0.05, use Krukal-Wallis Test
# Note: The Nemenyi test only accepts a factor independent variable so we add as.factor() to the indepe
kruskal.test(input_filt_rar$map_loaded$rich ~ input_filt_rar$map_loaded$Sample_type)
```

```
##
##  Kruskal-Wallis rank sum test
##
## data:  input_filt_rar$map_loaded$rich by input_filt_rar$map_loaded$Sample_type
## Kruskal-Wallis chi-squared = 9.2183, df = 2, p-value = 0.00996
```

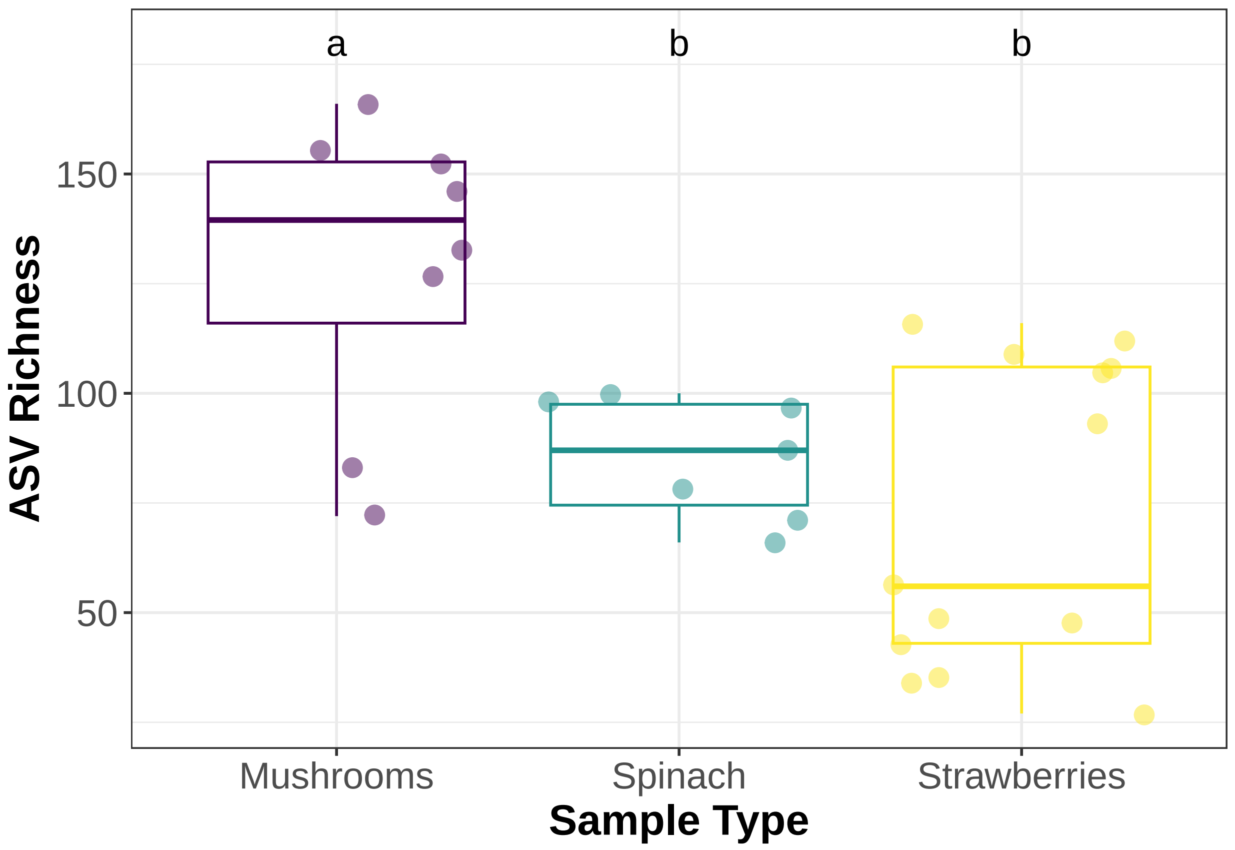
```r
# Significant effect. (agrees with ANOVA). Run posthoc.

kwAllPairsNemenyiTest(input_filt_rar$map_loaded$rich ~ as.factor(input_filt_rar$map_loaded$Sample_type))
```

```
##
##  Pairwise comparisons using Tukey-Kramer-Nemenyi all-pairs test with Tukey-Dist approximation

## data: input_filt_rar$map_loaded$rich by as.factor(input_filt_rar$map_loaded$Sample_type)

##              Mushrooms Spinach
## Spinach      0.0797    -
## Strawberries 0.0086    0.8879

##
## P value adjustment method: single-step

## alternative hypothesis: two.sided
```

```r
# Similar result, but spinach/mushroom different now only marginal. Use ANOVA result if assumptions pas

# Plot. Just copy the previous plot, change the variable name and the colour argument! We'll try the vi
ggplot(input_filt_rar$map_loaded, aes(Sample_type, rich, colour = Sample_type)) +
  geom_boxplot(outlier.shape = NA) +
  geom_jitter(size = 3, alpha = 0.5) +
  labs(x = "Sample Type", y = "ASV Richness", colour = "Farm Type") +
  scale_colour_viridis_d() +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14),
        legend.position = "none")
```



```r
# Now, since there was a significant difference, let's add some letter text. We'll make a new dataframe
label_df <- data.frame(x = c("Mushrooms", "Spinach", "Strawberries"),
                       y = c(180, 180, 180),
                       label = c("a", "b", "b"))

# Plot with added geom_text
ggplot(input_filt_rar$map_loaded, aes(Sample_type, rich, colour = Sample_type)) +
  geom_boxplot(outlier.shape = NA) +
  geom_jitter(size = 3, alpha = 0.5) +
  geom_text(data = label_df, aes(x, y, label = label), size = 5, inherit.aes = F) +
  labs(x = "Sample Type", y = "ASV Richness", colour = "Farm Type") +
  scale_colour_viridis_d() +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14),
        legend.position = "none")
```



```
# Multipanel plot.
# What one might want to show as, say, Figure 1 in a paper for an overview of the microbial communities
# Let's use an automated method for generating the significant difference letters, and then "melt" the

## Stats for rich and shannon using ANOVA and emmeans
m1 <- aov(rich ~ Sample_type, data = input_filt_rar$map_loaded)
t1 <- emmeans(object = m1, specs = "Sample_type") %>%
  cld(object = ., adjust = "Tukey", Letters = letters, alpha = 0.05) %>%
  mutate(name = "rich",
         y = 180)
```

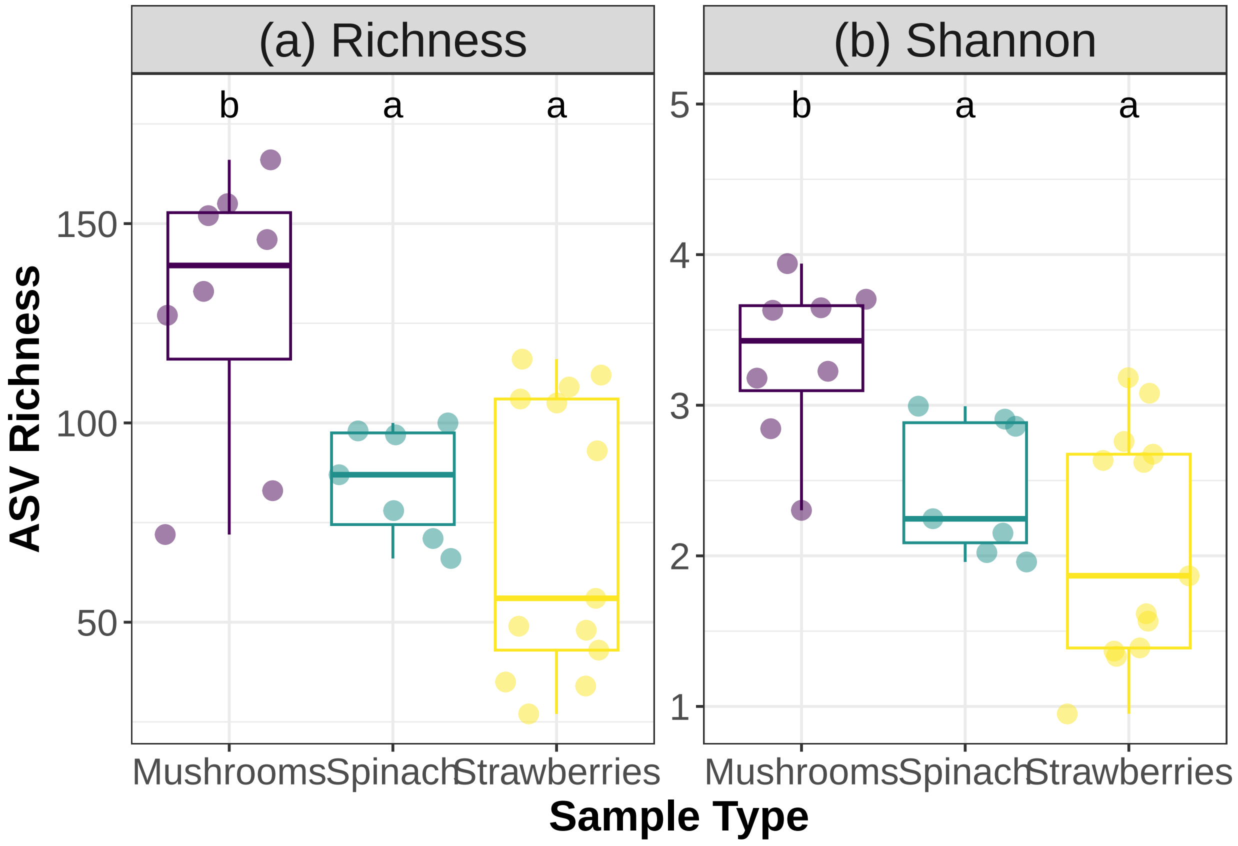
```
## Note: adjust = "tukey" was changed to "sidak"
## because "tukey" is only appropriate for one set of pairwise comparisons
```

```
m2 <- aov(shannon ~ Sample_type, data = input_filt_rar$map_loaded)
t2 <- emmeans(object = m2, specs = "Sample_type") %>%
  cld(object = ., adjust = "Tukey", Letters = letters, alpha = 0.05) %>%
  mutate(name = "shannon",
         y = 5)
```

```
## Note: adjust = "tukey" was changed to "sidak"
## because "tukey" is only appropriate for one set of pairwise comparisons
```

```
# Note: The sidak note is fine. It's still a Tukey posthoc test, just with sidak adjustment.

# Combine the labels
label_df <- rbind(t1, t2)
```

```
# Set names for the facet strips
facet_df <- c("rich" = "(a) Richness",
              "shannon" = "(b) Shannon")

# Make the data long format
alpha_long <- input_filt_rar$map_loaded %>%
  pivot_longer(cols = c("rich", "shannon"))
# This will put the richness and Shannon values into a column named "value"
# It will make a new column ("name") which maps the values to either richness or shannon

# Plot using facet_wrap to make the two panels. We use scales = "free_y" to let the y-axis scale vary a
ggplot(alpha_long, aes(Sample_type, value, colour = Sample_type)) +
  geom_boxplot(outlier.shape = NA) +
  geom_jitter(size = 3, alpha = 0.5) +
  geom_text(data = label_df, aes(Sample_type, y, label = str_trim(.group)), size = 5, inherit.aes = F) 
  labs(x = "Sample Type", y = "ASV Richness", colour = "Farm Type") +
  scale_colour_viridis_d() +
  facet_wrap(~name, ncol = 2, scales = "free_y", labeller = as_labeller(facet_df)) +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14),
        strip.text = element_text(size = 18),
        legend.position = "none")
```



BETA DIVERSITY

Aitchison and Bray-Curtis dissimilarity, ordinations, multivariate stats.

Bray-Curtis and NMDS

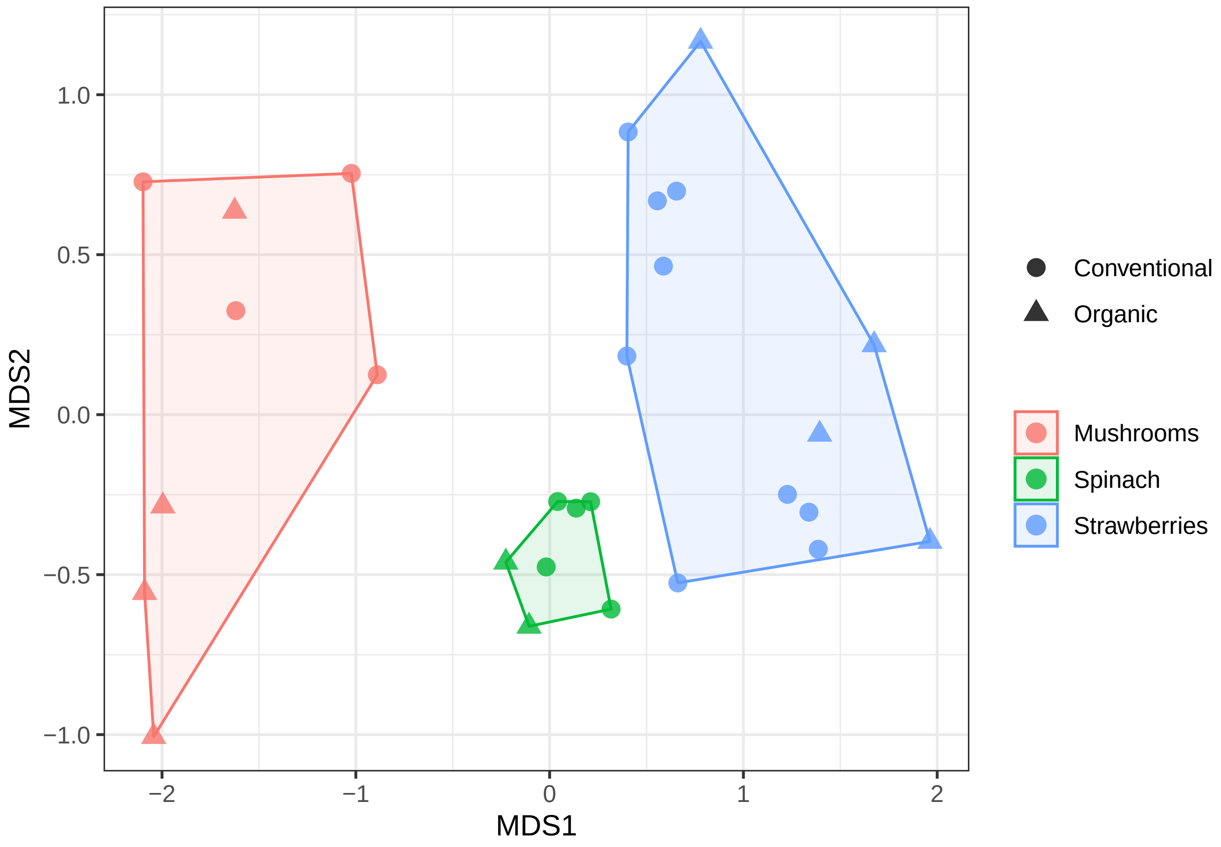
```
## The conventional method has long been to calculate a Bray-Curtis dissimilarity matrix and then plot

# Calculate dissimilarity matrix
dm <- calc_dm(input_filt_rar$data_loaded)
# NOTE: calc_dm uses Bray-Curtis by default. Can change to others using method=type_of_function in the

# Let's calculate an ordination from the distance matrix
ord <- calc_ordination(dm, 'nmds')
```

```
## Run 0 stress 0.1045557
## Run 1 stress 0.1227621
## Run 2 stress 0.1050761
## Run 3 stress 0.1045555
## ... New best solution
## ... Procrustes: rmse 0.0001632318  max resid 0.0006795909
## ... Similar to previous best
## Run 4 stress 0.1199644
## Run 5 stress 0.124056
## Run 6 stress 0.1050761
## Run 7 stress 0.1337521
## Run 8 stress 0.1199642
## Run 9 stress 0.1231582
## Run 10 stress 0.1240559
## Run 11 stress 0.1352062
## Run 12 stress 0.1199643
## Run 13 stress 0.1045556
## ... Procrustes: rmse 0.0003409372  max resid 0.001420896
## ... Similar to previous best
## Run 14 stress 0.1358001
## Run 15 stress 0.1045555
## ... Procrustes: rmse 0.0003152746  max resid 0.001313637
## ... Similar to previous best
## Run 16 stress 0.1249811
## Run 17 stress 0.1045555
## ... Procrustes: rmse 0.0003039307  max resid 0.001265566
## ... Similar to previous best
## Run 18 stress 0.1045556
## ... Procrustes: rmse 0.0003394067  max resid 0.001413822
## ... Similar to previous best
## Run 19 stress 0.1240559
## Run 20 stress 0.1240559
## *** Best solution repeated 5 times
```

```
# NOTE: You can also run pcoa and others by changing the code above from NMDS

# Now plot the ordination
plot_ordination(input_filt_rar, ord, 'Sample_type', 'Farm_type', hulls = TRUE)
```

```
## Warning: `do_()` was deprecated in dplyr 0.7.0.
## i Please use `do()` instead.
## i See vignette('programming') for more help
## i The deprecated feature was likely used in the mctoolsr package.
##   Please report the issue at <https://github.com/leffj/mctoolsr/issues>.
## This warning is displayed once every 8 hours.
## Call `lifecycle::last_lifecycle_warnings()` to see where this warning was
## generated.
```

```
## Warning: `group_by_()` was deprecated in dplyr 0.7.0.
## i Please use `group_by()` instead.
## i See vignette('programming') for more help
## i The deprecated feature was likely used in the mctoolsr package.
##   Please report the issue at <https://github.com/leffj/mctoolsr/issues>.
## This warning is displayed once every 8 hours.
## Call `lifecycle::last_lifecycle_warnings()` to see where this warning was
## generated.
```



```
#this is a nice thing to do at the start of an analysis to see what your data is doing

# NOTE: plot_ordination is a good way to quickly plot data. However, if you want to do more complicated
```

Bray-Curtis and PCoA

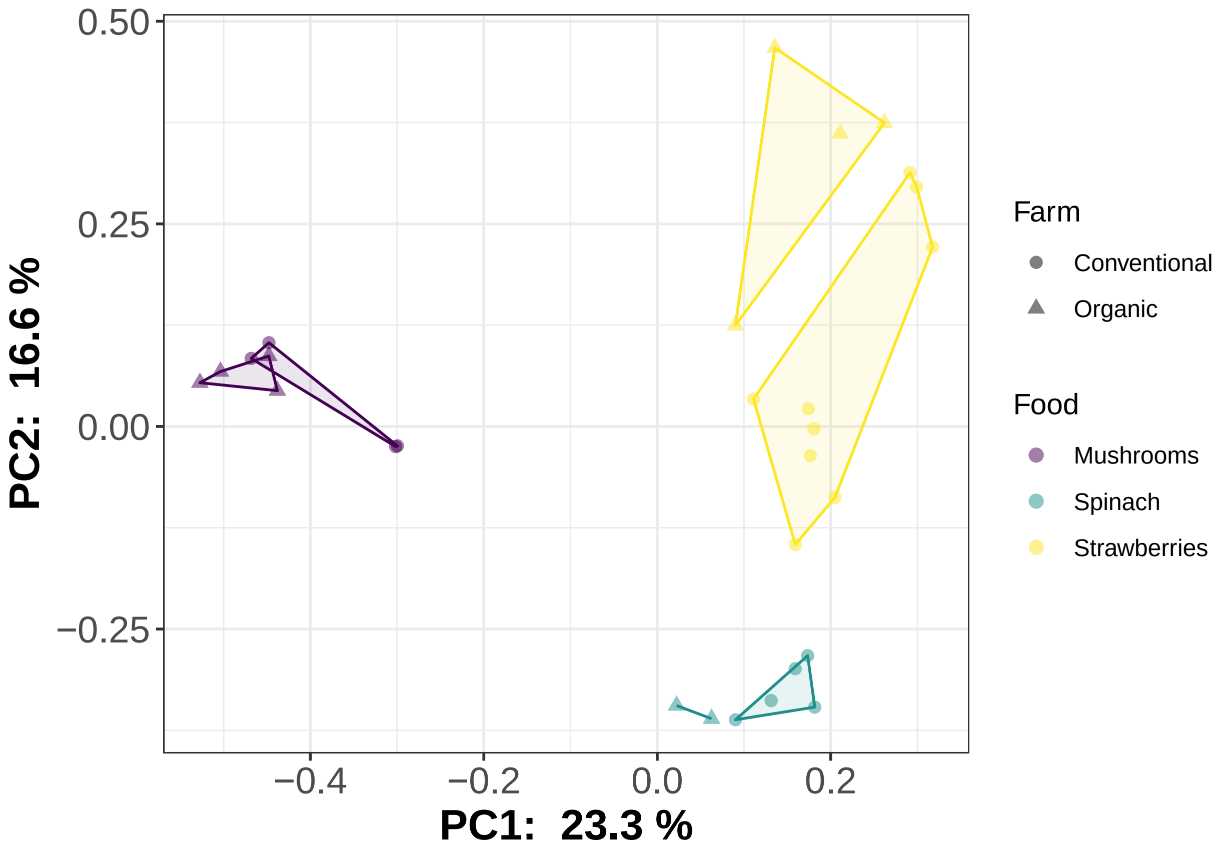
```
# Bray-Curtis dissimilarity matrix. Note: By default the data are square-root transformed.
bc <- calc_dm(input_filt_rar$data_loaded)

# Principle Coordinates Analysis (PCoA)
```

```r
pcoa <- cmdscale(bc, k = nrow(input_filt_rar$map_loaded) - 1, eig = T)

# Variation Explained
eigenvals(pcoa)/sum(eigenvals(pcoa)) # 23.3, 16.6 % variation explained
```

This does basically the same thing as above, but it uses Principle Coordinates Analysis (PCoA) instead of Non-metric multidimensional scaling (NMDS). It is worth seeing that you can do basically the same thing using two different codes and is a good check of the consistency of the ordination. We also demonstrate saving the ordination scores and making a custom ggplot figure.

```
##  [1] 0.23299874 0.16603055 0.12452778 0.08016005 0.04836278 0.04258608
##  [7] 0.03804139 0.03274082 0.02968823 0.02785019 0.02095102 0.02045540
## [13] 0.01652821 0.01518103 0.01370175 0.01275645 0.01188354 0.01128799
## [19] 0.00957814 0.00922398 0.00795294 0.00732115 0.00670822 0.00555404
## [25] 0.00428130 0.00314296 0.00050527 0.00000000
```

```r
# Make axis labels with % variation explained rounded to 1 digit.
pcoaA1 <- paste("PC1: ", round((eigenvals(pcoa)/sum(eigenvals(pcoa)))[1]*100, 1), "%")
pcoaA2 <- paste("PC2: ", round((eigenvals(pcoa)/sum(eigenvals(pcoa)))[2]*100, 1), "%")

# Save Axis 1 and 2 scores to the mapping file
input_filt_rar$map_loaded$Axis01 <- scores(pcoa)[,1]
input_filt_rar$map_loaded$Axis02 <- scores(pcoa)[,2]

# Function for making a convex hull
find_hull <- function(df) df[chull(df$Axis01, df$Axis02),]

# Calculate hulls and save to dataframe
micro.hulls <- ddply(input_filt_rar$map_loaded, c("Sample_type", "Farm_type"), find_hull)

# Plot
ggplot(input_filt_rar$map_loaded, aes(Axis01, Axis02, colour = Sample_type, shape = Farm_type)) +
  geom_polygon(data = micro.hulls, aes(colour = Sample_type, fill = Sample_type),
               alpha = 0.1, show.legend = F) +
  geom_point(size = 2, alpha = 0.5) +
  labs(x = pcoaA1,
       y = pcoaA2,
       colour = "Food",
       shape = "Farm") +
  scale_colour_viridis_d() +
  scale_fill_viridis_d() +
  theme_bw() +
  theme(legend.position = "right",
        axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14))
```



Aitchison Matrix and PCA

###Some authors (e.g., Gloor et al. 2017) have recently argued for using Aitchison's distance instead of Bray-Curtis dissimilarity. Then, a PCA instead of PCoA is performed. Instead of using rarefied data, we will use our unrarefied (but filtered) dataset and perform a center log ration transformation.

```
# First perform center log ratio transformation. Use filtered but unrarefied data for this ("input_filt
# CLR transformation
otu_czm <- cmultRepl(t(input_filt$data_loaded), label = 0, method = "CZM")
```

```
## Warning in cmultRepl(t(input_filt$data_loaded), label = 0, method = "CZM"): Column 1 containing more
## Column 2 containing more than 80% zeros/unobserved values was deleted (pre-check out using function 
## Column 3 containing more than 80% zeros/unobserved values was deleted (pre-check out using function 
## Column 4 containing more than 80% zeros/unobserved values was deleted (pre-check out using function 
## Column 6 containing more than 80% zeros/unobserved values was deleted (pre-check out using function 
## Column 7 containing more than 80% zeros/unobserved values was deleted (pre-check out using function 
## Column 8 containing more than 80% zeros/unobserved values was deleted (pre-check out using function 
## Column 10 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 11 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 12 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 13 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 14 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 15 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 16 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 17 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 18 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 19 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 20 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 21 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 22 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 24 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 25 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 26 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 28 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 29 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 30 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 31 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 32 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 34 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 35 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 36 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 37 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 38 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 39 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 40 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 41 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 42 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 43 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 44 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 45 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 47 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 48 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 49 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 51 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 52 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 53 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 55 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 56 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 57 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 59 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 61 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 62 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
## Column 63 containing more than 80% zeros/unobserved values was deleted (pre-check out using function
```

```
## Warning in cmultRepl(t(input_filt$data_loaded), label = 0, method = "CZM"): Row 3 containing more tha
## Row 17 containing more than 80% zeros/unobserved values was deleted (pre-check out using function zPa
## Row 19 containing more than 80% zeros/unobserved values was deleted (pre-check out using function zPa
## Row 20 containing more than 80% zeros/unobserved values was deleted (pre-check out using function zPa
## Row 26 containing more than 80% zeros/unobserved values was deleted (pre-check out using function zPa
```

```
## No. adjusted imputations:  84
```

```
otu_clr <- clr(otu_czm)
aclr <- compositions::dist(otu_clr)

# Filter dropped samples
# Columns and rows containing more than 80% zeros/unobserved values were deleted
# This is the bad thing about this method. We have now lost some samples and ASVs.
dim(t(input_filt$data_loaded)) # 28 samples, 933 ASVs
```

```
## [1]  28 933
```

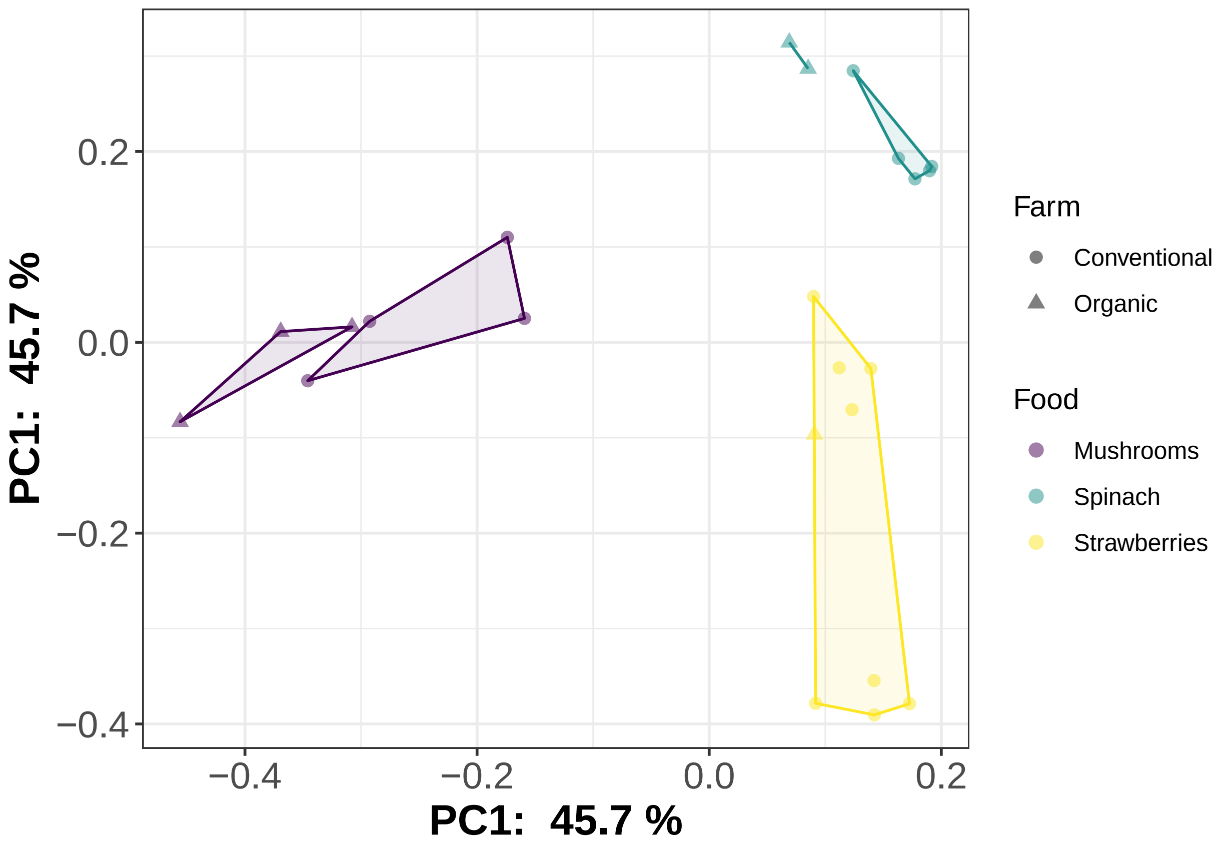
```r
dim(otu_czm) # 23 samples, 144 ASVs
```

```
## [1]  23 144
```

```r
# Make a sampleID column
input_filt$map_loaded$sampleID <- rownames(input_filt$map_loaded)

# Filter out sampleIDs not in the CLR dataset
input_filt_clr <- filter_data(input_filt,
                              filter_cat = "sampleID",
                              keep_vals = rownames(otu_czm))
```

```
## 23 samples remaining
```

```r
# PCA (principle components analysis)
d.pcx <- prcomp(aclr)

# % variation explained
d.mvar <- sum(d.pcx$sdev^2)

# Make axes labels with % variation explained
PC1 <- paste("PC1: ", round((sum(d.pcx$sdev[1]^2)/d.mvar)*100, 1), "%")
PC2 <- paste("PC2: ", round((sum(d.pcx$sdev[2]^2)/d.mvar)*100, 1), "%")

# Save scores to map_loaded dataframe
input_filt_clr$map_loaded$Axis01 <- d.pcx$x[,1]
input_filt_clr$map_loaded$Axis02 <- d.pcx$x[,2]

# Transform the scores
input_filt_clr$map_loaded$Axis01 <- input_filt_clr$map_loaded$Axis01/sqrt(sum((input_filt_clr$map_loade
input_filt_clr$map_loaded$Axis02 <- input_filt_clr$map_loaded$Axis02/sqrt(sum((input_filt_clr$map_loade

# Calculate hulls
micro.hulls <- ddply(input_filt_clr$map_loaded, c("Sample_type", "Farm_type"), find_hull)

# Plot
ggplot(input_filt_clr$map_loaded, aes(Axis01, Axis02, colour = Sample_type, shape = Farm_type)) +
  geom_polygon(data = micro.hulls, aes(colour = Sample_type, fill = Sample_type),
               alpha = 0.1, show.legend = F) +
  geom_point(size = 2, alpha = 0.5) +
  labs(x = PC1,
       y = PC1,
       colour = "Food",
       shape = "Farm") +
  scale_colour_viridis_d() +
  scale_fill_viridis_d() +
  theme_bw() +
  theme(legend.position = "right",
        axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14))
```



```
# Note: This is very consistent with the Bray-Curtis and PCoA.
```

PERMANOVA, PERMDISP

Now we need to run some statisitcs to test for differences in community composition among treatments as well as homogeneity of dispersion within treatments.

```
# PERMANOVA (multivariate version of ANOVA)
set.seed(1223) # To make reproducible
m <- adonis2(bc ~ input_filt_rar$map_loaded$Sample_type*input_filt_rar$map_loaded$Farm_type)
m
```

```
## Permutation test for adonis under reduced model
## Terms added sequentially (first to last)
## Permutation: free
## Number of permutations: 999
##
## adonis2(formula = bc ~ input_filt_rar$map_loaded$Sample_type * input_filt_rar$map_loaded$Farm_type)
##                                                                              Df
## input_filt_rar$map_loaded$Sample_type                                         2
## input_filt_rar$map_loaded$Farm_type                                           1
## input_filt_rar$map_loaded$Sample_type:input_filt_rar$map_loaded$Farm_type    2
## Residual                                                                     22
## Total                                                                        27
##                                                                              SumOfSqs
## input_filt_rar$map_loaded$Sample_type                                          3.4460
## input_filt_rar$map_loaded$Farm_type                                            0.4967
## input_filt_rar$map_loaded$Sample_type:input_filt_rar$map_loaded$Farm_type     0.8515
## Residual                                                                 4.7867
## Total                                                                    9.5809
##                                                                              R2
## input_filt_rar$map_loaded$Sample_type                                   0.35968
## input_filt_rar$map_loaded$Farm_type                                     0.05184
## input_filt_rar$map_loaded$Sample_type:input_filt_rar$map_loaded$Farm_type 0.08887
## Residual                                                                0.49961
## Total                                                                   1.00000
##                                                                               F
## input_filt_rar$map_loaded$Sample_type                                   7.9190
## input_filt_rar$map_loaded$Farm_type                                     2.2828
## input_filt_rar$map_loaded$Sample_type:input_filt_rar$map_loaded$Farm_type 1.9567
## Residual
## Total
##                                                                         Pr(>F)
## input_filt_rar$map_loaded$Sample_type                                    0.001
## input_filt_rar$map_loaded$Farm_type                                      0.013
## input_filt_rar$map_loaded$Sample_type:input_filt_rar$map_loaded$Farm_type  0.015
## Residual
## Total
##
## input_filt_rar$map_loaded$Sample_type                                   ***
## input_filt_rar$map_loaded$Farm_type                                     *
## input_filt_rar$map_loaded$Sample_type:input_filt_rar$map_loaded$Farm_type *
## Residual
## Total
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
# Significant effects of sample type, farm type and interaction.

# PERMDISP (multivariate version of Levene's Test)
m1 <- betadisper(bc, input_filt_rar$map_loaded$Sample_type)
anova(m1) # Dispersion different
```

```
## Analysis of Variance Table
##
## Response: Distances
##           Df   Sum Sq  Mean Sq F value    Pr(>F)
## Groups     2 0.146182 0.073091   20.36 5.663e-06 ***
## Residuals 25 0.089748 0.003590
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
m2 <- betadisper(bc, input_filt_rar$map_loaded$Farm_type)
anova(m2) # Dispersion homogeneous
```

```
## Analysis of Variance Table
##
## Response: Distances
##           Df  Sum Sq   Mean Sq F value Pr(>F)
## Groups     1 0.01824 0.0182398   2.586 0.1199
## Residuals 26 0.18339 0.0070533
```

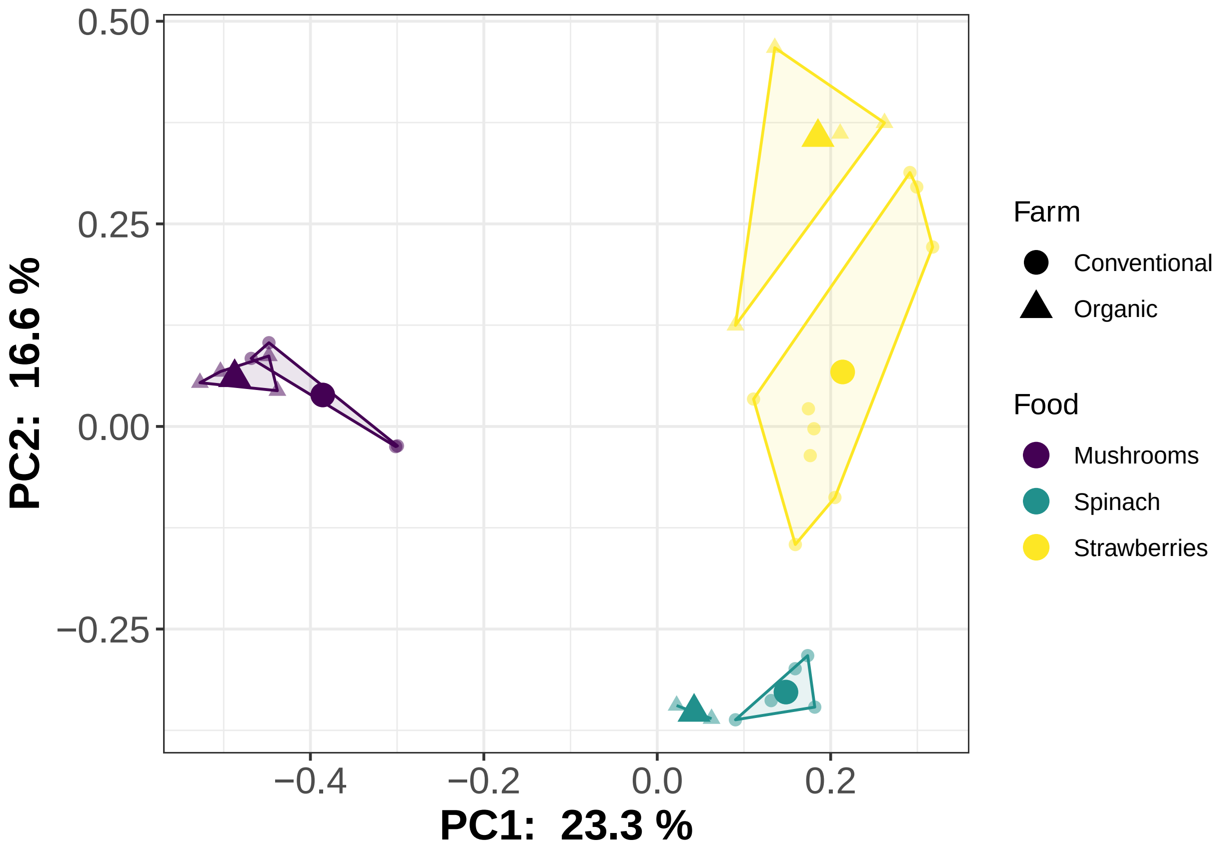
```
m3 <- betadisper(bc, input_filt_rar$map_loaded$Sample_Farming)
anova(m3) # Dispersion different
```

```
## Analysis of Variance Table
##
## Response: Distances
##           Df  Sum Sq  Mean Sq F value   Pr(>F)
## Groups     5 0.20207 0.040414  4.2705 0.007261 **
## Residuals 22 0.20820 0.009464
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
# Let's add the centroids to our PCoA plot. First make a clean data frame with %>%
m3$centroids
```

```
##                                PCoA1       PCoA2       PCoA3        PCoA4
## Mushrooms_Conventional    -0.38575907  0.03883206 -0.01061624 -0.262371754
## Mushrooms_Organic         -0.48736641  0.06046629  0.04946520  0.269786066
## Spinach_Conventional       0.14842443 -0.32777104  0.11029008  0.035217506
## Spinach_Organic            0.04248558 -0.35249459  0.16583474 -0.049806583
## Strawberries_Conventional  0.21382989  0.06724047 -0.11846836  0.005091353
## Strawberries_Organic       0.18535239  0.35687881  0.03360756  0.017843245
##                                 PCoA5        PCoA6       PCoA7        PCoA8
## Mushrooms_Conventional     0.007037599  0.010727625  0.01413591  0.024114352
## Mushrooms_Organic          0.045091973 -0.008263158  0.01704842 -0.018779568
## Spinach_Conventional       0.036795703  0.044499909 -0.10744589  0.057006371
## Spinach_Organic           -0.193589544 -0.136837666  0.09354917  0.009955471
## Strawberries_Conventional  0.067823499  0.022776835  0.06926734 -0.032714414
## Strawberries_Organic      -0.134110306 -0.046712483 -0.12714419 -0.016102192
##                                 PCoA9      PCoA10      PCoA11       PCoA12
## Mushrooms_Conventional    -0.006914227  0.11702031 -0.02698791  0.006258975
## Mushrooms_Organic         -0.007050640 -0.05766898  0.02147945  0.003249889
## Spinach_Conventional      -0.021404063  0.02237317  0.01008622  0.029165971
## Spinach_Organic            0.044903875 -0.07110968 -0.03133753 -0.042587096
## Strawberries_Conventional -0.034957121 -0.01011234 -0.01527359 -0.016338512
## Strawberries_Organic       0.040758276  0.02419418  0.03612080  0.024481711
##                                PCoA13       PCoA14        PCoA15       PCoA16
## Mushrooms_Conventional    -0.003960223 -0.006730886  0.0009626401  0.005563734
## Mushrooms_Organic          0.008540610 -0.005277012  0.0031150796 -0.005888112
## Spinach_Conventional       0.003465431 -0.036029157  0.0027143436  0.011429085
## Spinach_Organic           -0.026612229  0.091813095 -0.0083078665 -0.045376336
## Strawberries_Conventional -0.011999039  0.008081568 -0.0032411634 -0.000675784
## Strawberries_Organic       0.018272143  0.009819088  0.0021547380 -0.008962818
##                                PCoA17       PCoA18        PCoA19       PCoA20
## Mushrooms_Conventional     0.005720225  0.010544143 -2.115485e-05 -0.005627660
## Mushrooms_Organic          0.005028246 -0.008982367  6.953284e-03  0.010701290
## Spinach_Conventional      -0.007022220  0.015678956  6.312013e-03  0.013330204
## Spinach_Organic            0.034767559 -0.033496916 -2.966745e-02 -0.003740187
## Strawberries_Conventional -0.004949216 -0.008183587  4.035726e-03 -0.009357069
## Strawberries_Organic       0.012706439  0.009206557 -1.034710e-02  0.015367916
##                                 PCoA21       PCoA22        PCoA23       PCoA24
## Mushrooms_Conventional     0.0056129083  0.009042009  0.0009245449 -0.006018883
## Mushrooms_Organic         -0.0072696619  0.008235559  0.0006905917 -0.004527507
## Spinach_Conventional      -0.0097473366 -0.002722524  0.0052384694  0.019420532
## Spinach_Organic            0.0176757805  0.007474991 -0.0497621993 -0.021066493
## Strawberries_Conventional -0.0007101365  0.005141392 -0.0018611850 -0.001863626
## Strawberries_Organic      -0.0027793625 -0.011338879  0.0074435442 -0.006957195
##                                 PCoA25       PCoA26        PCoA27
## Mushrooms_Conventional    -0.0004846792 -0.001620327  0.0002772015
## Mushrooms_Organic          0.0024893278  0.001371614 -0.0001481248
## Spinach_Conventional       0.0012518818  0.003043980  0.0003390877
## Spinach_Organic           -0.0028499057  0.010268601 -0.0029428609
## Strawberries_Conventional -0.0037592705 -0.001903678  0.0030039133
## Strawberries_Organic       0.0117045356  0.002096310 -0.0060292168
```

```
centroids <- as.data.frame(m3$centroids) %>%
  dplyr::select(PCoA1, PCoA2) %>%
  rename(Axis01 = PCoA1,
         Axis02 = PCoA2) %>%
  rownames_to_column(var = "Sample_Farming") %>%
  separate(Sample_Farming, into = c("Sample_type", "Farm_type"), sep = "_")

# Remake the hulls
micro.hulls <- ddply(input_filt_rar$map_loaded, c("Sample_type", "Farm_type"), find_hull)

# Plot
ggplot(input_filt_rar$map_loaded, aes(Axis01, Axis02, colour = Sample_type, shape = Farm_type)) +
  geom_polygon(data = micro.hulls, aes(colour = Sample_type, fill = Sample_type),
               alpha = 0.1, show.legend = F) +
  geom_point(size = 2, alpha = 0.5) +
  geom_point(data = centroids, size = 4) +
  labs(x = pcoaA1,
       y = pcoaA2,
       colour = "Food",
       shape = "Farm") +
  scale_colour_viridis_d() +
  scale_fill_viridis_d() +
  theme_bw() +
  theme(legend.position = "right",
        axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14))
```



Multipanel PCoA plots

```
# Bonus: Let's make a multipanel PCoA plot. This can be really useful if you have a 16S dataset and an

# Bray-Curtis Matrix
input_nolet <- filter_data(input,
                           filter_cat = "Sample_type",
                           filter_vals = "Lettuce") # 28 samples remaining (removed 4)
```

```
## 28 samples remaining
```

```
bc2 <- calc_dm(input_nolet$data_loaded)

# Principle Coordinates Analysis (PCoA)
pcoa2 <- cmdscale(bc2, k = nrow(input_nolet$map_loaded) - 1, eig = T)

# Variation Explained
eigenvals(pcoa2)/sum(eigenvals(pcoa2)) # 18.0, 13.9 % variation explained
```

```
##  [1] 0.18038793 0.13909543 0.10727935 0.06995555 0.04782424 0.04251012
##  [7] 0.04037428 0.03387551 0.03344651 0.03081348 0.02753299 0.02585268
## [13] 0.02451780 0.02207721 0.02006282 0.01860184 0.01722138 0.01578966
## [19] 0.01516671 0.01493567 0.01433535 0.01329144 0.01264790 0.01010843
## [25] 0.00947210 0.00716815 0.00565548 0.00000000
```

```
# Save Axis 1 and 2 to the mapping file
input_nolet$map_loaded$Axis01 <- scores(pcoa2)[,1]
input_nolet$map_loaded$Axis02 <- scores(pcoa2)[,2]
```

```r
# Now we need to combine the two datasets. We'll stack them on top of each other with rbind. In order t
df1 <- input_filt_rar$map_loaded %>%
  dplyr::select(Sample_type, Farm_type, Sample_Farming, Axis01, Axis02) %>%
  mutate(Dataset = "a) Rarefied, filtered")
df2 <- input_nolet$map_loaded %>%
  dplyr::select(Sample_type, Farm_type, Sample_Farming, Axis01, Axis02) %>%
  mutate(Dataset = "b) Unrarefied, unfiltered")
df3 <- rbind(df1, df2)

# Now we'll make a label data frame for the % variation explained. To get the right x and y values, che
range(df1$Axis01)
```

```
## [1] -0.5274262  0.3176546
```

```r
range(df2$Axis01)
```

```
## [1] -0.4816218  0.2969321
```

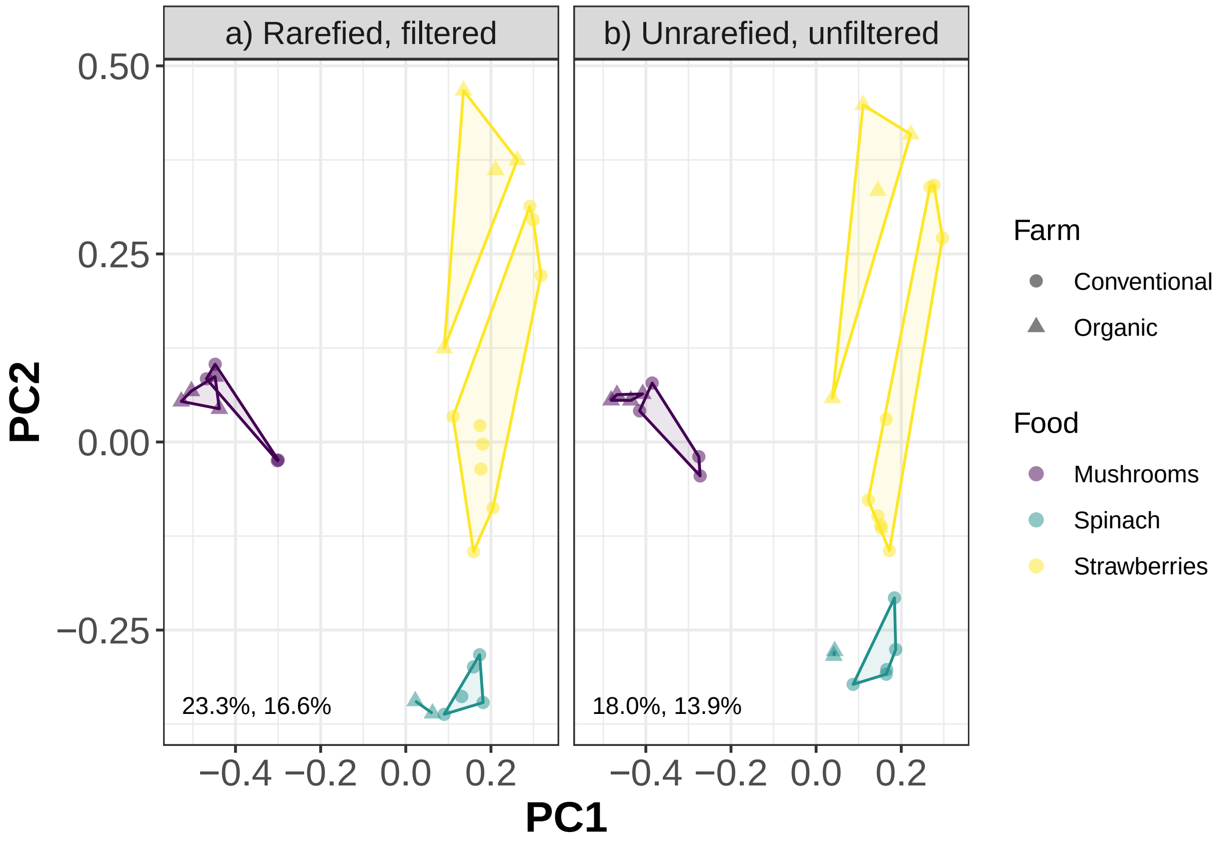
```r
range(df1$Axis02)
```

```
## [1] -0.3619609  0.4673646
```

```r
range(df2$Axis02)
```

```
## [1] -0.3221893  0.4481281
```

```r
label_df2 <- data.frame(x = c(-0.35, -0.35),
                        y = c(-0.35, -0.35),
                        Dataset = c("a) Rarefied, filtered", "b) Unrarefied, unfiltered"),
                        label = c("23.3%, 16.6%", "18.0%, 13.9%"))

# Now we need to make new hulls. The hulls will be the same as before but we'll add Dataset as a variab
micro.hulls2 <- ddply(df3, c("Dataset","Sample_type","Farm_type"), find_hull)
ggplot(df3, aes(Axis01, Axis02, colour = Sample_type, shape = Farm_type)) +
  geom_polygon(data = micro.hulls2, aes(colour = Sample_type, fill = Sample_type),
               alpha = 0.1, show.legend = F) +
  geom_point(size = 2, alpha = 0.5) +
  geom_text(data = label_df2, aes(x, y, label = label), size = 3, inherit.aes = F) +
  labs(x = "PC1",
       y = "PC2",
       colour = "Food",
       shape = "Farm") +
  scale_colour_viridis_d() +
  scale_fill_viridis_d() +
  facet_wrap(~ Dataset) + # We did not define a scales argument, so axes have the same scales
  theme_bw() +
  theme(legend.position = "right",
        axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14),
        strip.text = element_text(size = 12))
```

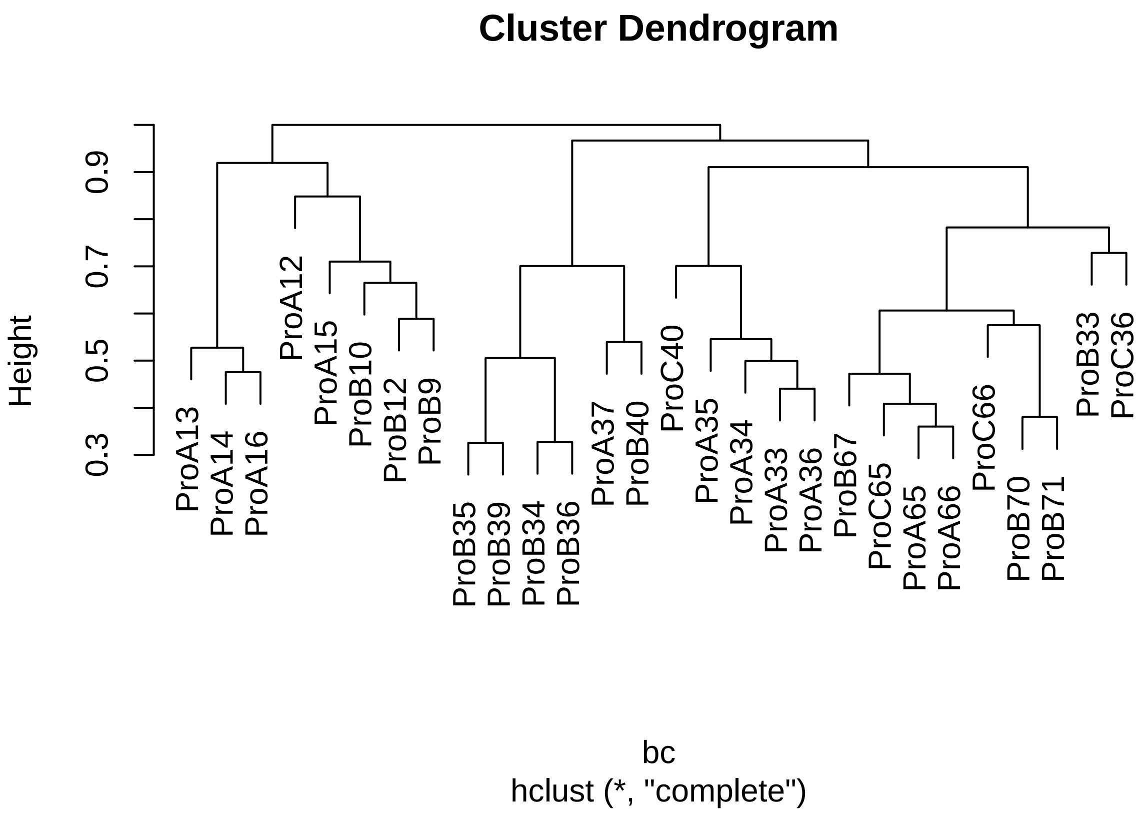


```
# The overall trends are the same but there were some minor shifts. Also the rarefied/filtered dataset
```

Hierarchical Clustering

Another option to examine community composition among your samples and treatments is to see how they cluster. You can already see how they cluster in the ordination plots, but this is another method that can be used.

```
# Cluster according to Ward's D2. There are other methods available.
# The input is a dissimilarity matrix. Here we'll use the Bray-Curtis one.
h <- hclust(bc)

# The default plot of the saved hclust object will immediately give you an overview of how the samples
plot(h)
```



TAXONOMIC ANALYSIS

Indicator taxa - SIMPER or MULTIPATT - NOTE: This takes up a large amount of computer memory and can sometimes take a while or max out your computer. It will run faster/better on a server or if you just run it on the most abundant taxa instead of tens of thousands of ASVs. In this case, this is a simple dataset and we won't have any problems.

```
# SIMPER (list how much each ASV contributes to dissimilarity among groups)
sim <- simper(t(input_filt_rar$data_loaded),
              input_filt_rar$map_loaded$Sample_type)
s <- summary(sim)
# Let's look at the top 5 contributing to dissimilarity between mushrooms and strawberries/spinach
head(s$Mushrooms_Strawberries, n = 5)
```

```
##               average         sd     ratio     ava          avb    cumsum     p
## OTU_5339 0.15510254 0.15712746 0.9871129   0.000 294.38461538 0.1579854 0.035
## OTU_1763 0.07767285 0.10876583 0.7141292   0.750 147.53846154 0.2371020 0.205
## OTU_7240 0.07004843 0.04265811 1.6420893 133.875   0.92307692 0.3084524 0.001
## OTU_6975 0.05174982 0.05932183 0.8723571   3.625 101.30769231 0.3611641 1.000
## OTU_8870 0.04310712 0.06974072 0.6181054  81.875   0.07692308 0.4050725 0.001
```

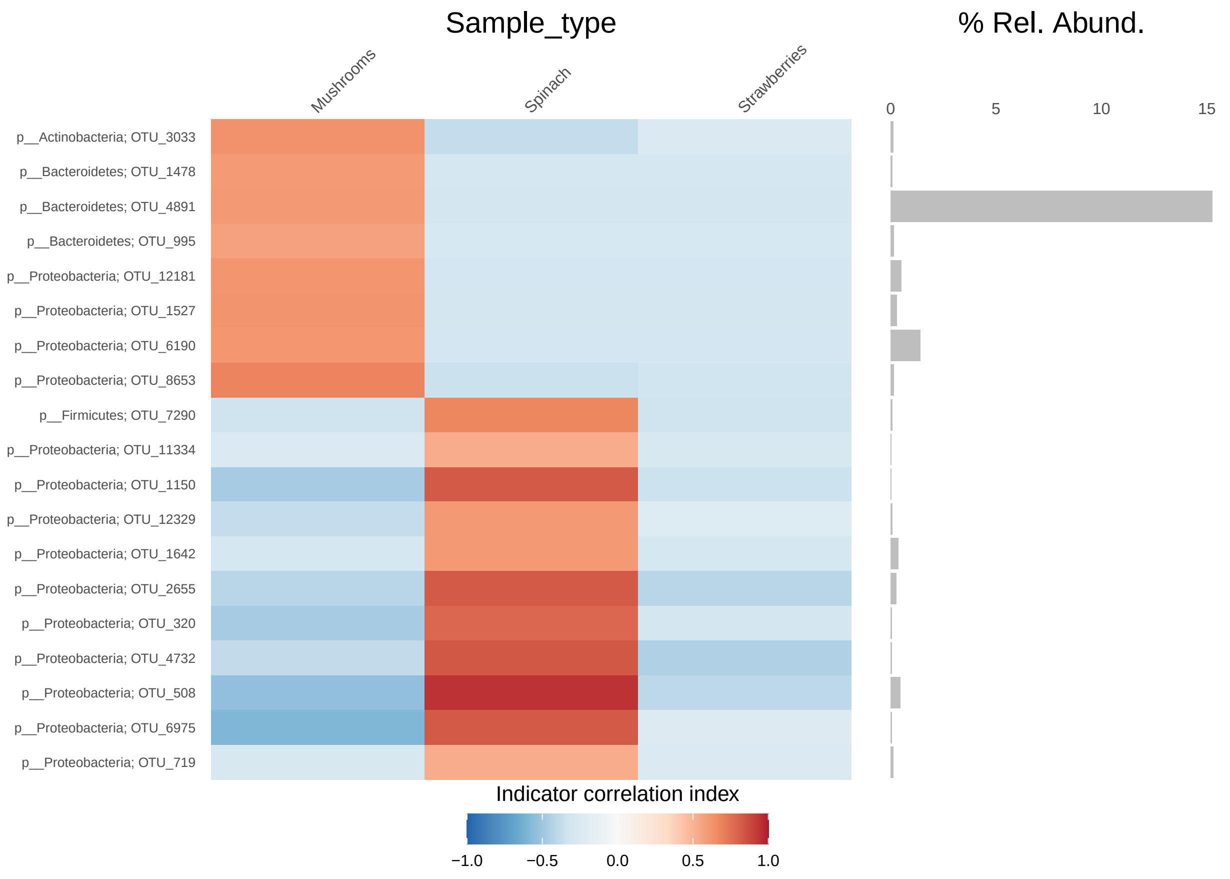
```
head(s$Mushrooms_Spinach, n = 5)
```

```
##               average         sd     ratio     ava       avb    cumsum     p
## OTU_6975 0.20191367 0.06950675 2.9049506   3.625 386.85714 0.2172342 0.001
## OTU_6245 0.06529429 0.04392433 1.4865178   5.500 129.42857 0.2874828 0.001
## OTU_7240 0.05378782 0.04099386 1.3120945 133.875  44.57143 0.3453519 0.038
## OTU_8870 0.04277999 0.06993490 0.6117117  81.875   1.00000 0.3913778 0.025
## OTU_9155 0.03562961 0.05730298 0.6217759  67.625   0.00000 0.4297109 0.005
```

```
# average is the proportion contribution, cumsum is cumulative, ava and avb are mean sequence abundance
# in this case it looks like the top 5 ASVs are expalining a lot of the difference (~40%)

# MULTIPATT (list ASVs associated with each group)
# Groups can be individual treatments or groups of treatments
set.seed(1223) # For reproducability
mp <- multipatt(t(input_filt_rar$data_loaded),
                input_filt_rar$map_loaded$Sample_type,
                func = "IndVal.g",
                control = how(nperm=999))
summary(mp)
```

```
##
##  Multilevel pattern analysis
##  ---------------------------
##
##  Association function: IndVal.g
##  Significance level (alpha): 0.05
##
##  Total number of species: 888
##  Selected number of species: 204
##  Number of species associated to 1 group: 191
##  Number of species associated to 2 groups: 13
##
##  List of species associated to each combination:
##
##  Group Mushrooms  #sps.  107
##           stat p.value
## OTU_7703  0.999   0.001 ***
## OTU_4891  0.935   0.001 ***
## OTU_6190  0.935   0.001 ***
## OTU_7523  0.935   0.001 ***
## OTU_9155  0.935   0.001 ***
## OTU_8870  0.929   0.004 **
## OTU_3033  0.877   0.001 ***
## OTU_3636  0.866   0.001 ***
## OTU_8177  0.866   0.001 ***
## OTU_12181 0.866   0.001 ***
## OTU_8653  0.858   0.001 ***
## OTU_3785  0.846   0.002 **
## OTU_1478  0.791   0.001 ***
## OTU_1527  0.791   0.001 ***
## OTU_1730  0.791   0.004 **
## OTU_3432  0.791   0.002 **
## OTU_4062  0.791   0.001 ***
## OTU_6747  0.791   0.002 **
## OTU_8874  0.791   0.002 **
## OTU_9608  0.791   0.002 **
## OTU_10514 0.791   0.001 ***
## OTU_11482 0.791   0.002 **
## OTU_11952 0.791   0.002 **
## OTU_12614 0.791   0.001 ***
## OTU_6760  0.790   0.002 **
## OTU_5927  0.779   0.011 *
## OTU_2395  0.776   0.002 **
## OTU_13366 0.742   0.006 **
## OTU_995   0.707   0.001 ***
## OTU_1295  0.707   0.004 **
## OTU_2241  0.707   0.007 **
## OTU_2402  0.707   0.006 **
## OTU_2428  0.707   0.004 **
## OTU_2797  0.707   0.006 **
## OTU_3563  0.707   0.004 **
## OTU_3804  0.707   0.005 **
## OTU_4354  0.707   0.006 **
## OTU_4456  0.707   0.006 **
## OTU_4688  0.707   0.007 **
## OTU_5186  0.707   0.006 **
## OTU_7569  0.707   0.004 **
## OTU_9903  0.707   0.003 **
## OTU_10146 0.707   0.006 **
## OTU_10284 0.707   0.008 **
## OTU_10759 0.707   0.006 **
## OTU_10769 0.707   0.006 **
## OTU_11299 0.707   0.006 **
## OTU_12424 0.707   0.005 **
## OTU_12968 0.707   0.004 **
## OTU_5354  0.697   0.009 **
## OTU_10979 0.690   0.021 *
## OTU_9742  0.644   0.041 *
## OTU_229   0.612   0.031 *
## OTU_242   0.612   0.028 *
## OTU_294   0.612   0.026 *
## OTU_378   0.612   0.028 *
## OTU_860   0.612   0.023 *
## OTU_1029  0.612   0.028 *
## OTU_1160  0.612   0.024 *
## OTU_1542  0.612   0.025 *
## OTU_1702  0.612   0.024 *
## OTU_1709  0.612   0.028 *
## OTU_1931  0.612   0.025 *
## OTU_2039  0.612   0.028 *
## OTU_2270  0.612   0.034 *
## OTU_2608  0.612   0.028 *
## OTU_2798  0.612   0.027 *
## OTU_2900  0.612   0.028 *
## OTU_2953  0.612   0.029 *
## OTU_3052  0.612   0.031 *
## OTU_3189  0.612   0.025 *
## OTU_3888  0.612   0.028 *
## OTU_4413  0.612   0.028 *
## OTU_5096  0.612   0.024 *
## OTU_5444  0.612   0.032 *
## OTU_5510  0.612   0.026 *
## OTU_6041  0.612   0.034 *
## OTU_6059  0.612   0.030 *
## OTU_6387  0.612   0.023 *
## OTU_6535  0.612   0.027 *
## OTU_6801  0.612   0.028 *
## OTU_8687  0.612   0.028 *
## OTU_8801  0.612   0.028 *
## OTU_8859  0.612   0.028 *
## OTU_9313  0.612   0.028 *
## OTU_9890  0.612   0.032 *
## OTU_10038 0.612   0.024 *
## OTU_10315 0.612   0.028 *
## OTU_10477 0.612   0.028 *
## OTU_10500 0.612   0.028 *
## OTU_10656 0.612   0.027 *
## OTU_11129 0.612   0.031 *
## OTU_11269 0.612   0.026 *
## OTU_11456 0.612   0.028 *
## OTU_11562 0.612   0.031 *
## OTU_11584 0.612   0.037 *
## OTU_11773 0.612   0.026 *
## OTU_12146 0.612   0.030 *
## OTU_12885 0.612   0.031 *
## OTU_13134 0.612   0.034 *
## OTU_13180 0.612   0.026 *
## OTU_13398 0.612   0.032 *
## OTU_1844  0.603   0.022 *
## OTU_11078 0.599   0.038 *
## OTU_1882  0.587   0.037 *
## OTU_7606  0.587   0.042 *
## OTU_8713  0.583   0.043 *
##
##  Group Spinach  #sps.  58
##           stat p.value
## OTU_2655  1.000   0.001 ***
## OTU_4732  0.978   0.001 ***
## OTU_8281  0.972   0.001 ***
## OTU_1150  0.955   0.001 ***
## OTU_508   0.951   0.001 ***
## OTU_320   0.939   0.001 ***
## OTU_719   0.913   0.001 ***
## OTU_8799  0.886   0.001 ***
## OTU_1642  0.845   0.001 ***
## OTU_7290  0.845   0.001 ***
## OTU_13238 0.831   0.001 ***
## OTU_11334 0.823   0.001 ***
## OTU_4398  0.818   0.002 **
## OTU_10433 0.796   0.012 *
## OTU_10513 0.789   0.002 **
## OTU_12329 0.787   0.001 ***
## OTU_1618  0.778   0.002 **
## OTU_12015 0.773   0.001 ***
## OTU_3608  0.756   0.003 **
## OTU_7030  0.756   0.002 **
## OTU_9070  0.756   0.003 **
## OTU_11070 0.756   0.004 **
## OTU_12275 0.756   0.002 **
## OTU_9708  0.718   0.003 **
## OTU_7733  0.715   0.014 *
## OTU_5443  0.710   0.009 **
## OTU_2146  0.697   0.007 **
## OTU_1626  0.655   0.012 *
## OTU_2178  0.655   0.006 **
## OTU_2250  0.655   0.013 *
## OTU_3148  0.655   0.011 *
## OTU_3695  0.655   0.014 *
## OTU_3696  0.655   0.012 *
## OTU_5320  0.655   0.016 *
## OTU_5514  0.655   0.010 **
## OTU_5578  0.655   0.005 **
## OTU_7854  0.655   0.012 *
## OTU_9378  0.655   0.017 *
## OTU_10070 0.655   0.016 *
## OTU_11796 0.655   0.010 **
## OTU_10845 0.631   0.005 **
## OTU_3614  0.631   0.022 *
## OTU_6661  0.628   0.019 *
## OTU_1960  0.622   0.024 *
## OTU_2617  0.620   0.025 *
## OTU_7365  0.603   0.025 *
## OTU_367   0.535   0.050 *
## OTU_1821  0.535   0.044 *
## OTU_3843  0.535   0.043 *
## OTU_4741  0.535   0.045 *
## OTU_7297  0.535   0.050 *
## OTU_7881  0.535   0.043 *
## OTU_8958  0.535   0.043 *
## OTU_10494 0.535   0.050 *
## OTU_11077 0.535   0.050 *
## OTU_12457 0.535   0.050 *
## OTU_12665 0.535   0.050 *
## OTU_12730 0.535   0.045 *
##
##  Group Strawberries  #sps.  26
##            stat p.value
## OTU_2080  0.784   0.003 **
## OTU_1763  0.781   0.011 *
## OTU_989   0.734   0.009 **
## OTU_3427  0.734   0.012 *
## OTU_4804  0.734   0.014 *
## OTU_7411  0.734   0.012 *
## OTU_5339  0.733   0.009 **
## OTU_10680 0.731   0.015 *
## OTU_10922 0.730   0.012 *
## OTU_9761  0.714   0.029 *
## OTU_2502  0.679   0.019 *
## OTU_4013  0.679   0.021 *
## OTU_4304  0.679   0.018 *
## OTU_6219  0.638   0.041 *
## OTU_6817  0.638   0.034 *
## OTU_13306 0.635   0.038 *
## OTU_6225  0.632   0.035 *
## OTU_370   0.620   0.024 *
## OTU_1683  0.620   0.048 *
## OTU_1689  0.620   0.031 *
## OTU_5122  0.620   0.035 *
## OTU_6276  0.620   0.037 *
## OTU_7337  0.620   0.036 *
## OTU_8990  0.620   0.034 *
## OTU_10876 0.620   0.041 *
## OTU_12316 0.620   0.035 *
##
##  Group Mushrooms+Spinach  #sps.  4
##            stat p.value
## OTU_7240  0.997   0.001 ***
## OTU_13300 0.816   0.002 **
## OTU_5323  0.775   0.004 **
## OTU_5568  0.666   0.037 *
##
##  Group Spinach+Strawberries  #sps.  9
##            stat p.value
## OTU_6975  0.996   0.001 ***
## OTU_6245  0.984   0.001 ***
## OTU_11811 0.952   0.001 ***
## OTU_64    0.894   0.001 ***
## OTU_1275  0.806   0.001 ***
## OTU_7928  0.806   0.002 **
## OTU_9230  0.707   0.033 *
## OTU_908   0.671   0.047 *
## OTU_13190 0.671   0.049 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```r
# Now lets use a custom function for plotting just 1 group indicators and choosing p value and indicato
# Rerun the multipatt with the r.g func.
set.seed(1223) # For reproducability
mp <- multipatt(t(input_filt_rar$data_loaded),
                input_filt_rar$map_loaded$Sample_type,
                func = "r.g",
                control = how(nperm=999))

# This function was built with an 8th taxonomy column containing the ASV ID. Make here.
input_filt_rar$taxonomy_loaded$taxonomy8 <- rownames(input_filt_rar$taxonomy_loaded)

# It takes a summarized taxonomy dataframe, made like this.
tax_sum_asv <- summarize_taxonomy(input_filt_rar, level = 8, report_higher_tax = FALSE)

# The group must be a factor
input_filt_rar$map_loaded$Sample_type <- as.factor(input_filt_rar$map_loaded$Sample_type)

# Run the function.
plot_multipatt_asv(mp_obj = mp,
                   input = input_filt_rar,
                   tax_sum = tax_sum_asv,
                   group = "Sample_type",
                   filter = FALSE,
                   abund = "% Rel. Abund.",
                   qcut = 0.05, # Adjusted p value< 0.05
                   rcut = 0.5) # Can change r cutoff to be more or less selective
```



```
# This will plot the ASVs that passed the cutoffs. It will plot how correlated they are with a group as
```

##OTU/ASV Specific functions

```
# Let's look at the top taxa across samples (Can change the number you want seen)
return_top_taxa(input = input_filt_rar, number_taxa = 10)
```

```
##              taxonomy1         taxonomy2               taxonomy3
## OTU_6975   k__Bacteria p__Proteobacteria c__Gammaproteobacteria
## OTU_5339   k__Bacteria p__Proteobacteria c__Gammaproteobacteria
## OTU_1763   k__Bacteria     p__Firmicutes              c__Bacilli
## OTU_6245   k__Bacteria p__Proteobacteria c__Gammaproteobacteria
## OTU_7240   k__Bacteria p__Proteobacteria c__Gammaproteobacteria
## OTU_8870   k__Bacteria  p__Bacteroidetes       c__Flavobacteriia
## OTU_9155   k__Bacteria  p__Bacteroidetes     c__Sphingobacteriia
## OTU_10680 k__Bacteria p__Proteobacteria c__Alphaproteobacteria
## OTU_5927   k__Bacteria  p__Bacteroidetes     c__Sphingobacteriia
## OTU_320    k__Bacteria p__Proteobacteria c__Gammaproteobacteria
##                         taxonomy4                taxonomy5           taxonomy6
## OTU_6975    o__Enterobacteriales  f__Enterobacteriaceae          g__Erwinia
## OTU_5339   o__Enterobacteriales  f__Enterobacteriaceae         g__Buchnera
## OTU_1763          o__Bacillales         f__Bacillaceae                 g__
## OTU_6245   o__Enterobacteriales  f__Enterobacteriaceae                 g__
## OTU_7240     o__Pseudomonadales    f__Pseudomonadaceae      g__Pseudomonas
## OTU_8870    o__Flavobacteriales     f__[Weeksellaceae] g__Chryseobacterium
## OTU_9155  o__Sphingobacteriales f__Sphingobacteriaceae       g__Pedobacter
## OTU_10680   o__Sphingomonadales   f__Sphingomonadaceae     g__Sphingomonas
## OTU_5927  o__Sphingobacteriales f__Sphingobacteriaceae g__Sphingobacterium
## OTU_320    o__Enterobacteriales  f__Enterobacteriaceae          g__Erwinia
##            taxonomy7 taxonomy8
## OTU_6975         s__  OTU_6975
## OTU_5339         s__  OTU_5339
## OTU_1763         s__  OTU_1763
## OTU_6245         s__  OTU_6245
## OTU_7240         s__  OTU_7240
## OTU_8870         s__  OTU_8870
## OTU_9155         s__  OTU_9155
## OTU_10680        s__ OTU_10680
## OTU_5927  s__faecium  OTU_5927
## OTU_320          s__   OTU_320
```

```
# Calculates the top taxa based on number of counts - taking the row means for each OTU

# What taxa are common? Give them rarefied input, then level = "what level we want to summarize our inf
tax_sum_phyla <- summarize_taxonomy(input_filt_rar, level = 2, report_higher_tax = FALSE)
#level taxa=false- just look at that level, not at whole taxonomic classification

# How many phyla can we detect in this dataset
tax_sum_phyla[1:5, 1:8]
```

```
##                       ProA12      ProA13      ProA14      ProA15      ProA16
## p__[Thermi]        0.0000000 0.000000000 0.000000000 0.000000000 0.000000000
## p__Actinobacteria  0.4130664 0.006322445 0.002107482 0.023182297 0.003161222
## p__Armatimonadetes 0.0000000 0.000000000 0.000000000 0.001053741 0.000000000
## p__Bacteroidetes   0.1022129 0.576396207 0.871443625 0.405690200 0.622760801
## p__Chlamydiae      0.0000000 0.000000000 0.000000000 0.003161222 0.000000000
##                       ProA33      ProA34      ProA35
## p__[Thermi]        0.000000000 0.006322445 0.000000000
## p__Actinobacteria  0.042149631 0.066385669 0.094836670
## p__Armatimonadetes 0.000000000 0.000000000 0.000000000
## p__Bacteroidetes   0.001053741 0.001053741 0.002107482
## p__Chlamydiae      0.000000000 0.000000000 0.000000000
```

```
# Give most abundant phyla across dataset
tax_sum_phyla_Means <- sort(rowMeans(tax_sum_phyla), decreasing = T)
tax_sum_phyla_Means[1:5]
```

```
## p__Proteobacteria  p__Bacteroidetes     p__Firmicutes p__Actinobacteria
##       0.722038236       0.135142255       0.110680415       0.029730543
##    p__Chloroflexi
##       0.001392443
```

```
# Now lets try summarizing at the family level (level 5)
tax_sum_families <- summarize_taxonomy(input_filt_rar, level = 5, report_higher_tax = FALSE)
head(tax_sum_families)
```

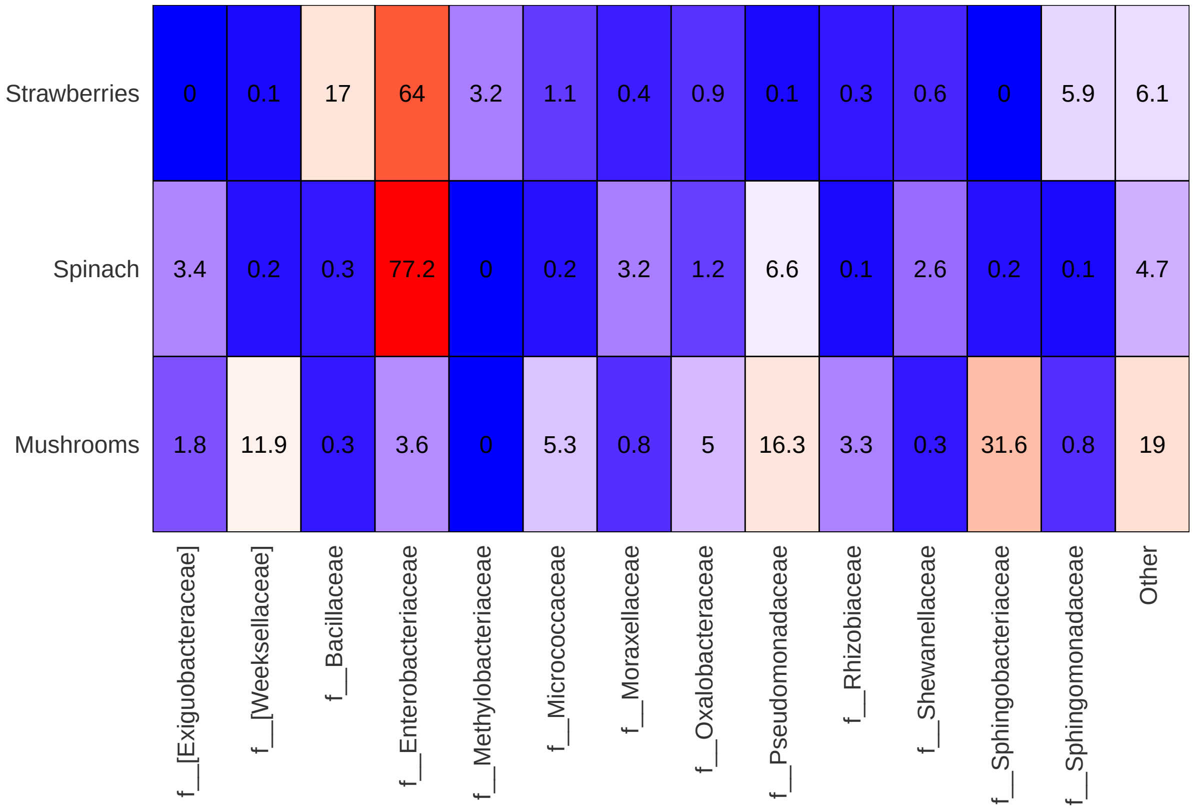
```
##                            ProA12      ProA13      ProA14      ProA15
## f__                   0.012644889 0.002107482 0.001053741 0.038988409
## f__[Chromatiaceae]     0.002107482 0.000000000 0.000000000 0.002107482
## f__[Exiguobacteraceae] 0.053740780 0.002107482 0.000000000 0.000000000
## f__[Fimbriimonadaceae] 0.000000000 0.000000000 0.000000000 0.001053741
## f__[Weeksellaceae]     0.010537408 0.081138040 0.516332982 0.005268704
## f__Acetobacteraceae    0.000000000 0.000000000 0.000000000 0.000000000
##                          ProA16      ProA33      ProA34      ProA35
## f__                   0.0000000 0.008429926 0.002107482 0.002107482
## f__[Chromatiaceae]     0.0000000 0.000000000 0.000000000 0.000000000
## f__[Exiguobacteraceae] 0.0000000 0.000000000 0.000000000 0.000000000
## f__[Fimbriimonadaceae] 0.0000000 0.000000000 0.000000000 0.000000000
## f__[Weeksellaceae]     0.1928346 0.001053741 0.000000000 0.001053741
## f__Acetobacteraceae    0.0000000 0.022128556 0.001053741 0.000000000
##                            ProA36 ProA37      ProA65      ProA66      ProB10
## f__                   0.002107482      0 0.000000000 0.000000000 0.014752371
## f__[Chromatiaceae]     0.000000000      0 0.000000000 0.000000000 0.005268704
## f__[Exiguobacteraceae] 0.000000000      0 0.033719705 0.051633298 0.007376185
## f__[Fimbriimonadaceae] 0.000000000      0 0.000000000 0.000000000 0.000000000
## f__[Weeksellaceae]     0.000000000      0 0.003161222 0.001053741 0.106427819
## f__Acetobacteraceae    0.000000000      0 0.000000000 0.000000000 0.000000000
##                            ProB12      ProB33     ProB34      ProB35
## f__                   0.006322445 0.000000000 0.00000000 0.000000000
## f__[Chromatiaceae]     0.053740780 0.001053741 0.00000000 0.000000000
## f__[Exiguobacteraceae] 0.012644889 0.000000000 0.00000000 0.000000000
## f__[Fimbriimonadaceae] 0.000000000 0.000000000 0.00000000 0.000000000
## f__[Weeksellaceae]     0.011591149 0.002107482 0.00000000 0.000000000
## f__Acetobacteraceae    0.000000000 0.028451001 0.02212856 0.006322445
##                           ProB36     ProB39      ProB40      ProB67
## f__                   0.00000000 0.00000000 0.000000000 0.000000000
## f__[Chromatiaceae]     0.00000000 0.00000000 0.000000000 0.000000000
## f__[Exiguobacteraceae] 0.00000000 0.00000000 0.000000000 0.027397260
## f__[Fimbriimonadaceae] 0.00000000 0.00000000 0.000000000 0.000000000
## f__[Weeksellaceae]     0.00000000 0.01159115 0.000000000 0.002107482
## f__Acetobacteraceae    0.01791359 0.00000000 0.001053741 0.000000000
##                            ProB70      ProB71       ProB9      ProC36
## f__                   0.002107482 0.000000000 0.004214963 0.004214963
## f__[Chromatiaceae]     0.000000000 0.000000000 0.024236038 0.000000000
## f__[Exiguobacteraceae] 0.057955743 0.061116965 0.068493151 0.002107482
## f__[Fimbriimonadaceae] 0.000000000 0.000000000 0.000000000 0.000000000
## f__[Weeksellaceae]     0.003161222 0.003161222 0.024236038 0.000000000
## f__Acetobacteraceae    0.000000000 0.000000000 0.000000000 0.000000000
##                            ProC40      ProC65      ProC66
## f__                   0.001053741 0.000000000 0.000000000
## f__[Chromatiaceae]     0.000000000 0.000000000 0.000000000
## f__[Exiguobacteraceae] 0.000000000 0.005268704 0.002107482
## f__[Fimbriimonadaceae] 0.000000000 0.000000000 0.000000000
## f__[Weeksellaceae]     0.000000000 0.000000000 0.000000000
## f__Acetobacteraceae    0.000000000 0.000000000 0.000000000
```

Other useful plotting and analysis functions from base mctoolsR as well as some customizations

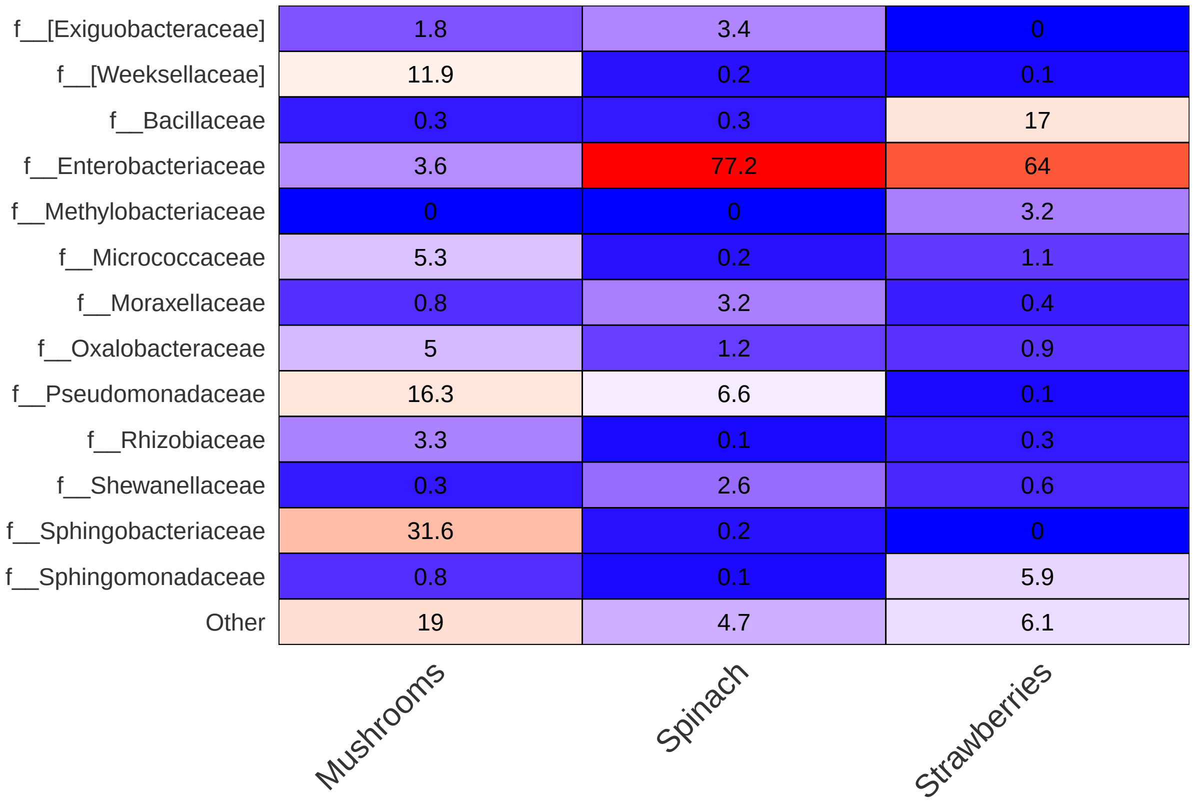
For More information on mctoolsR functions see tutorial at https://github.com/leffj/mctoolsr

```
# Summarize at family level. Choose different levels with the level argument
tax_sum_families <- summarize_taxonomy(input_filt_rar, level = 5, report_higher_tax = FALSE)

# Default heatmap
plot_ts_heatmap(tax_sum_families,
                input_filt_rar$map_loaded,
                0.01,
                'Sample_type')
```

```
## Warning: `mutate_()` was deprecated in dplyr 0.7.0.
## i Please use `mutate()` instead.
## i See vignette('programming') for more help
## i The deprecated feature was likely used in the mctoolsr package.
##   Please report the issue at <https://github.com/leffj/mctoolsr/issues>.
## This warning is displayed once every 8 hours.
## Call `lifecycle::last_lifecycle_warnings()` to see where this warning was
## generated.
```



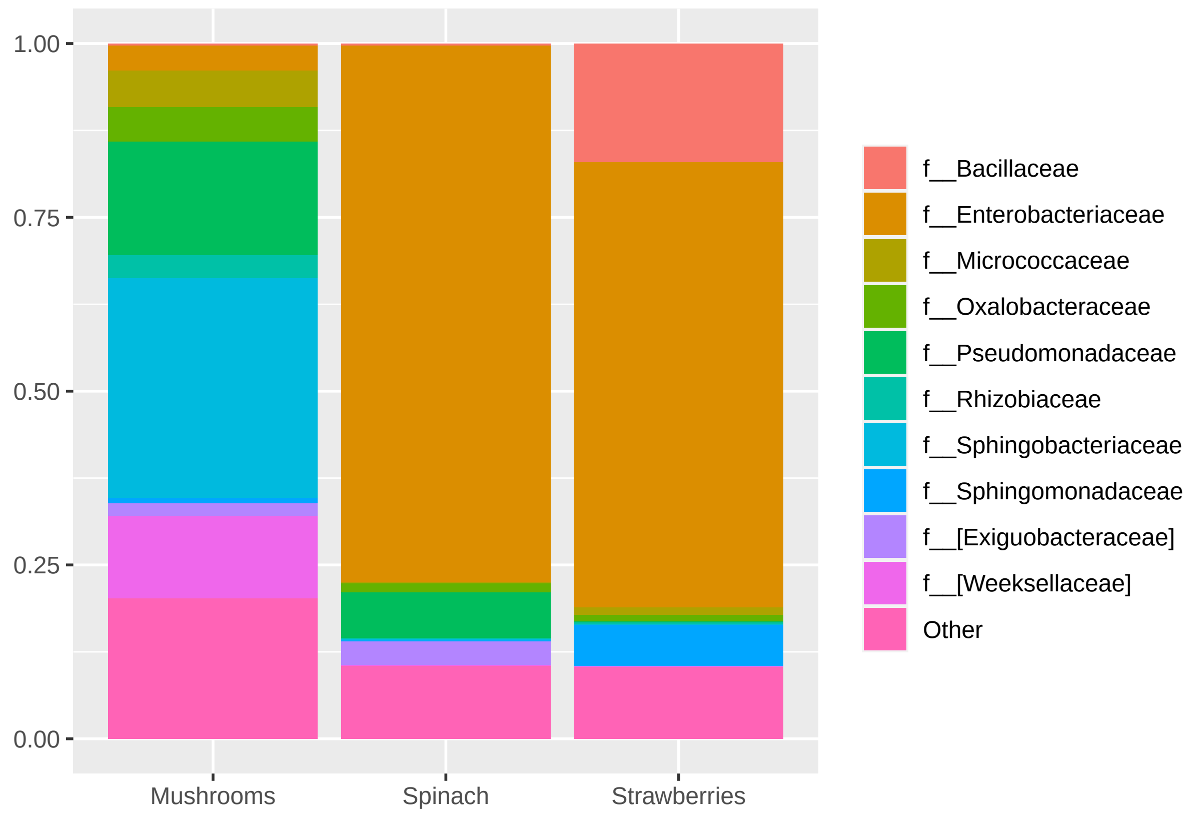
```
# Add some customization
plot_ts_heatmap(tax_sum_families,
                input_filt_rar$map_loaded,
                0.01,
                'Sample_type',
                rev_taxa = T) +
  coord_flip() +
  theme(axis.text.x = element_text(size = 12, angle = 45, vjust = 1))
```



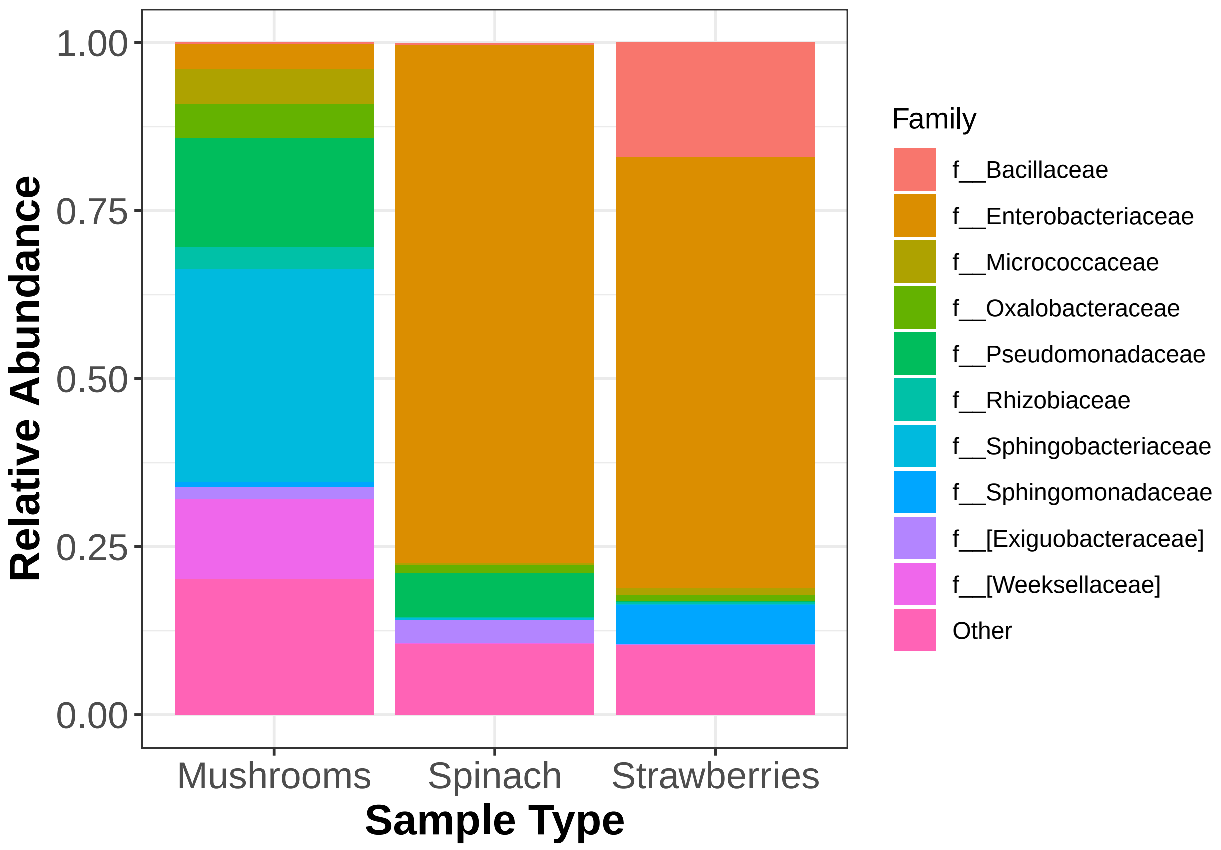
	Mushrooms	Spinach	Strawberries
f__[Exiguobacteraceae]	1.8	3.4	0
f__[Weeksellaceae]	11.9	0.2	0.1
f__Bacillaceae	0.3	0.3	17
f__Enterobacteriaceae	3.6	77.2	64
f__Methylobacteriaceae	0	0	3.2
f__Micrococcaceae	5.3	0.2	1.1
f__Moraxellaceae	0.8	3.2	0.4
f__Oxalobacteraceae	5	1.2	0.9
f__Pseudomonadaceae	16.3	6.6	0.1
f__Rhizobiaceae	3.3	0.1	0.3
f__Shewanellaceae	0.3	2.6	0.6
f__Sphingobacteriaceae	31.6	0.2	0
f__Sphingomonadaceae	0.8	0.1	5.9
Other	19	4.7	6.1

```
# Default (choose how many taxa you want with num_taxa)
plot_taxa_bars(tax_sum_families,
               input_filt_rar$map_loaded,
               "Sample_type",
               num_taxa = 10)
```

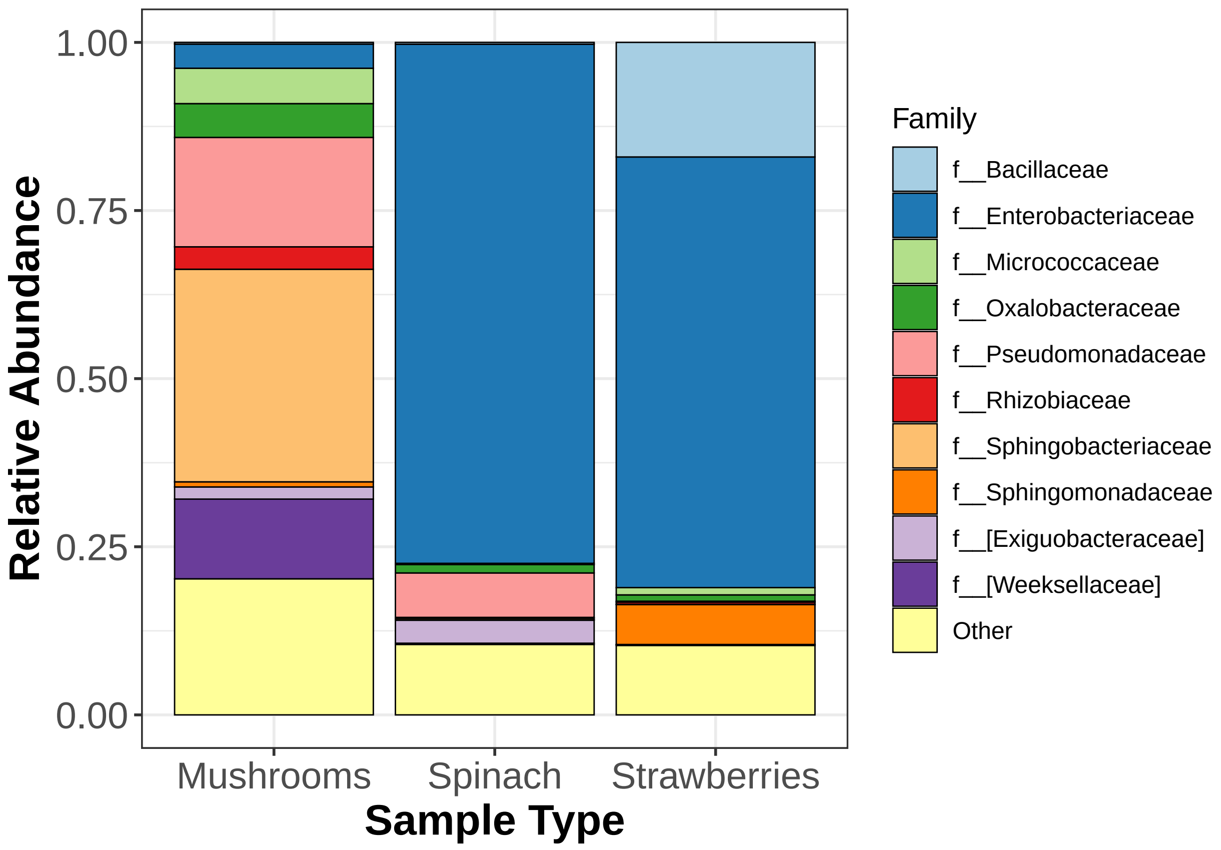
```
## Warning: `summarise_()` was deprecated in dplyr 0.7.0.
## i Please use `summarise()` instead.
## i The deprecated feature was likely used in the mctoolsr package.
##   Please report the issue at <https://github.com/leffj/mctoolsr/issues>.
## This warning is displayed once every 8 hours.
## Call `lifecycle::last_lifecycle_warnings()` to see where this warning was
## generated.
```



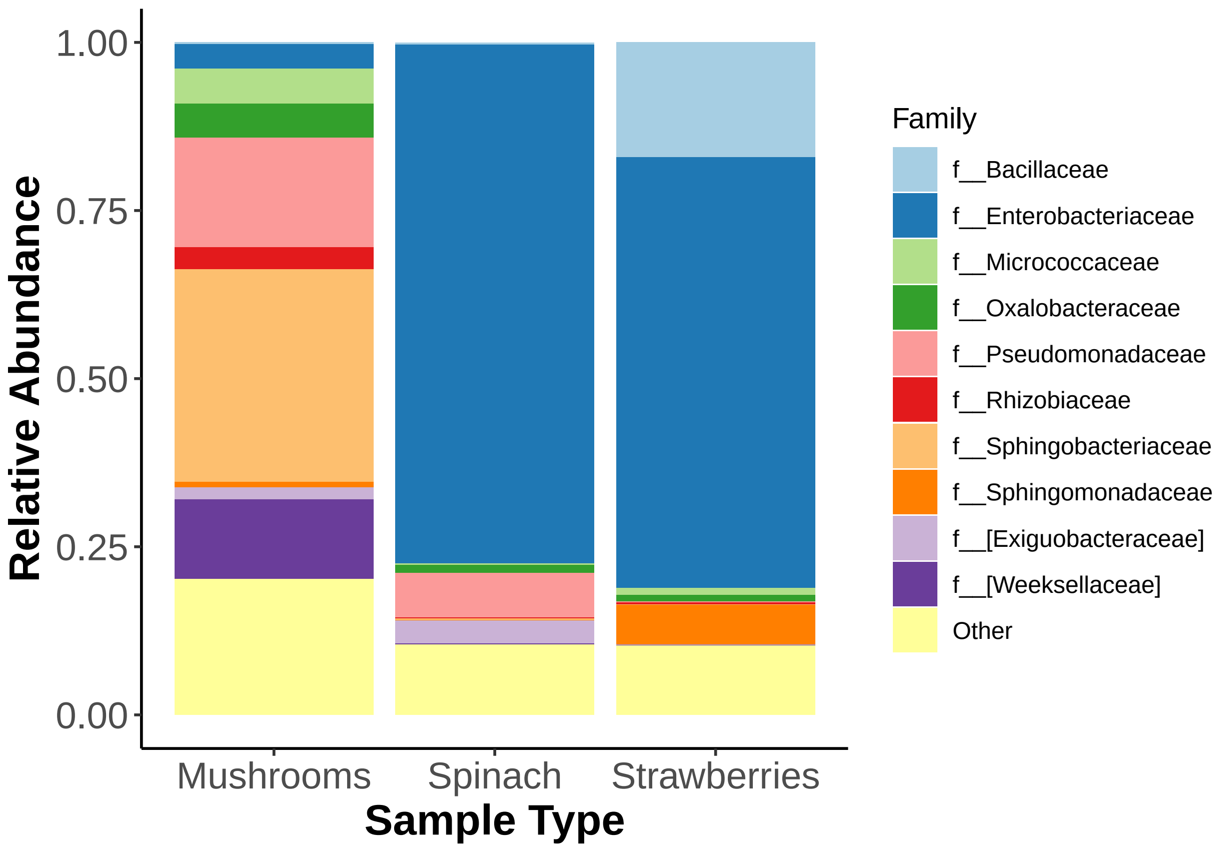
```r
# Add some customization
plot_taxa_bars(tax_sum_families,
               input_filt_rar$map_loaded,
               "Sample_type",
               num_taxa = 10) +
  labs(x = "Sample Type", y = "Relative Abundance", fill = "Family") +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14))
```



```r
# Add even more customization. plot_taxa_bars has a data_only argument. So you can save the dataset cre
bars <- plot_taxa_bars(tax_sum_families,
                       input_filt_rar$map_loaded,
                       "Sample_type",
                       num_taxa = 10,
                       data_only = TRUE)
ggplot(bars, aes(group_by, mean_value, fill = taxon)) +
  geom_bar(stat = "identity", colour = "black", linewidth = 0.25) +
  labs(x = "Sample Type", y = "Relative Abundance", fill = "Family") +
  scale_fill_brewer(palette = "Paired") +
  theme_bw() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14))
```

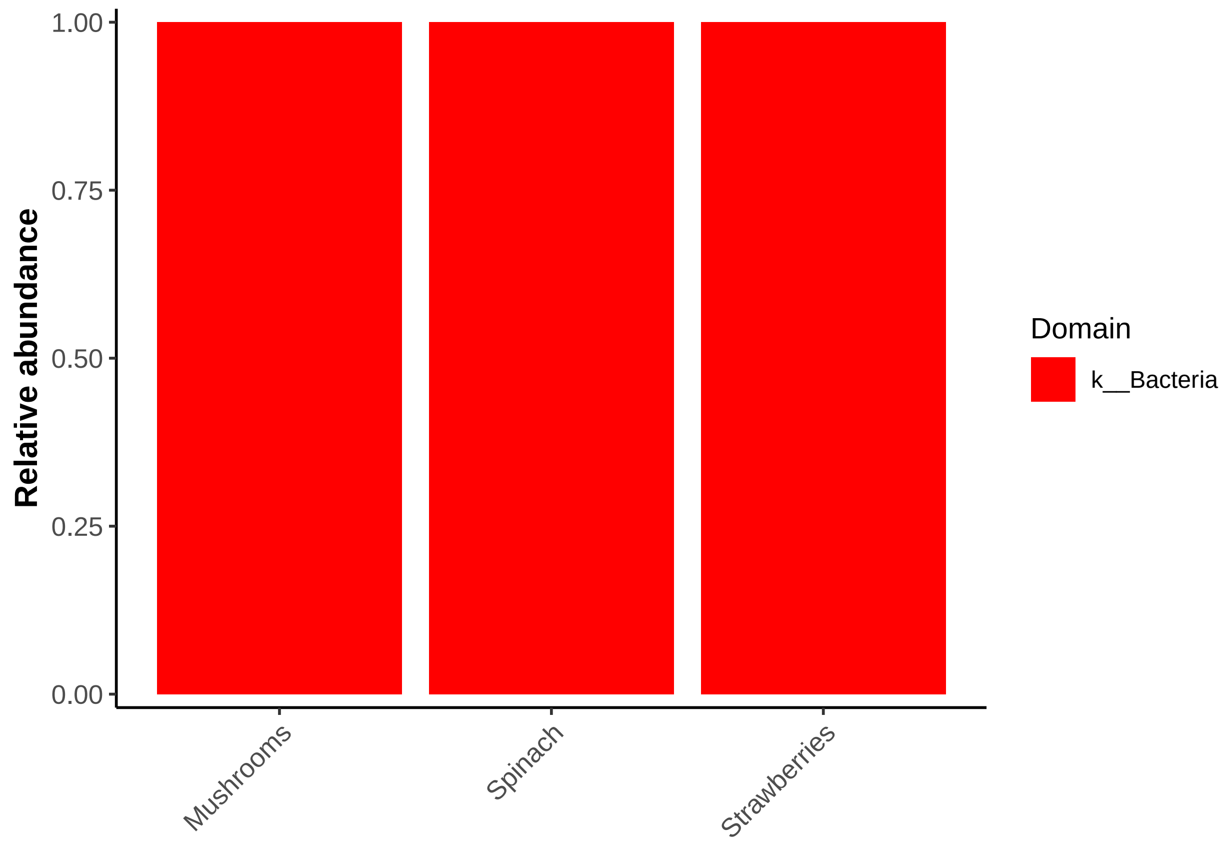


```r
# Different style - classic theme, no black lines between taxa.
ggplot(bars, aes(group_by, mean_value, fill = taxon)) +
  geom_bar(stat = "identity", linewidth = 0.25) +
  labs(x = "Sample Type", y = "Relative Abundance", fill = "Family") +
  scale_fill_brewer(palette = "Paired") +
  theme_classic() +
  theme(axis.title = element_text(face = "bold", size = 16),
        axis.text = element_text(size = 14))
```

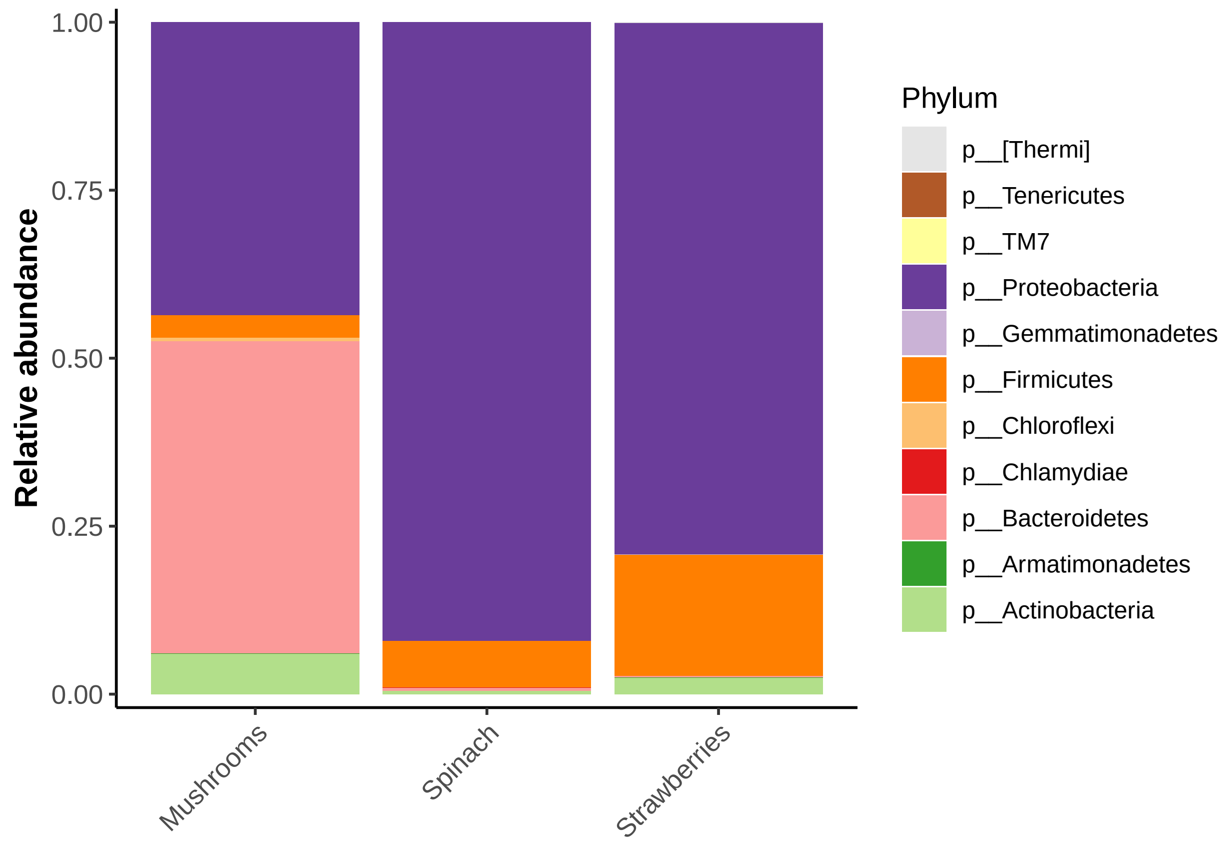


```
# Now try a cliffplot! This is a custom function by Cliff Bueno de Mesquita that serves as a nice but q
cliffplot_taxa_bars(input = input_filt_rar, level = 1, variable = "Sample_type")
```

```
## Warning: Using `size` aesthetic for lines was deprecated in ggplot2 3.4.0.
## i Please use `linewidth` instead.
## This warning is displayed once every 8 hours.
## Call `lifecycle::last_lifecycle_warnings()` to see where this warning was
## generated.
```

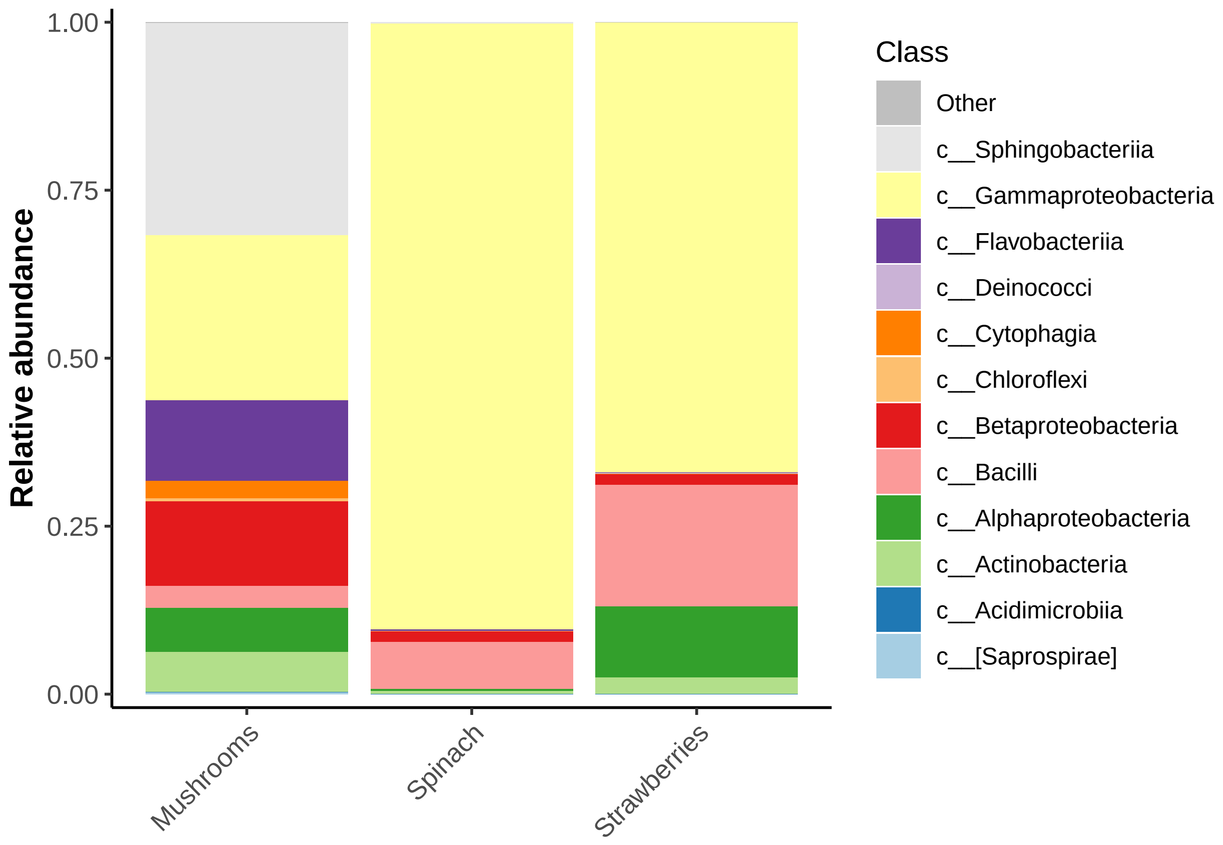


```r
cliffplot_taxa_bars(input = input_filt_rar, level = 2, variable = "Sample_type")
```



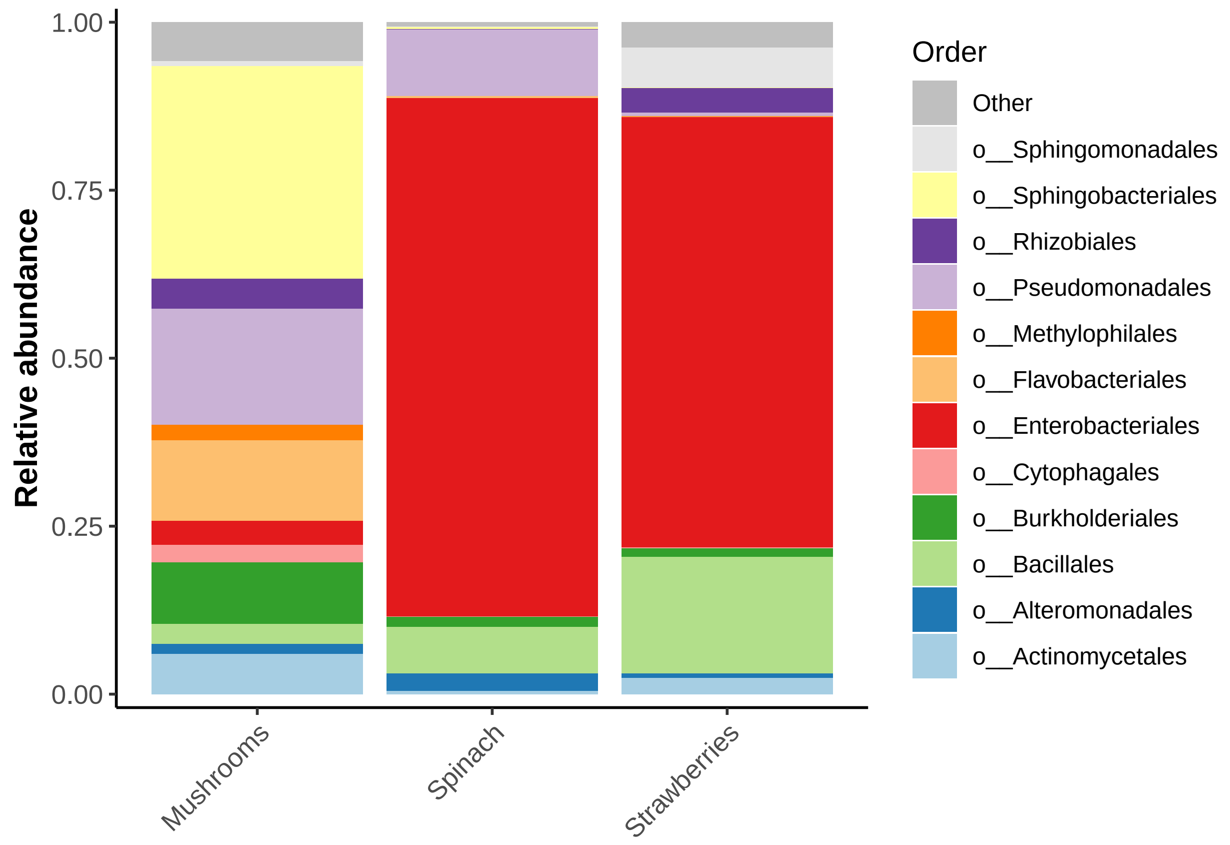
```r
cliffplot_taxa_bars(input = input_filt_rar, level = 3, variable = "Sample_type")
```

```
## Warning: There were 3 warnings in `mutate()`.
## The first warning was:
## i In argument: `taxon = fct_relevel(taxon, "Unclassified", after = Inf)`.
## i In group 1: `group_by = Mushrooms`.
## Caused by warning:
## ! 1 unknown level in `f`: Unclassified
## i Run `dplyr::last_dplyr_warnings()` to see the 2 remaining warnings.
```



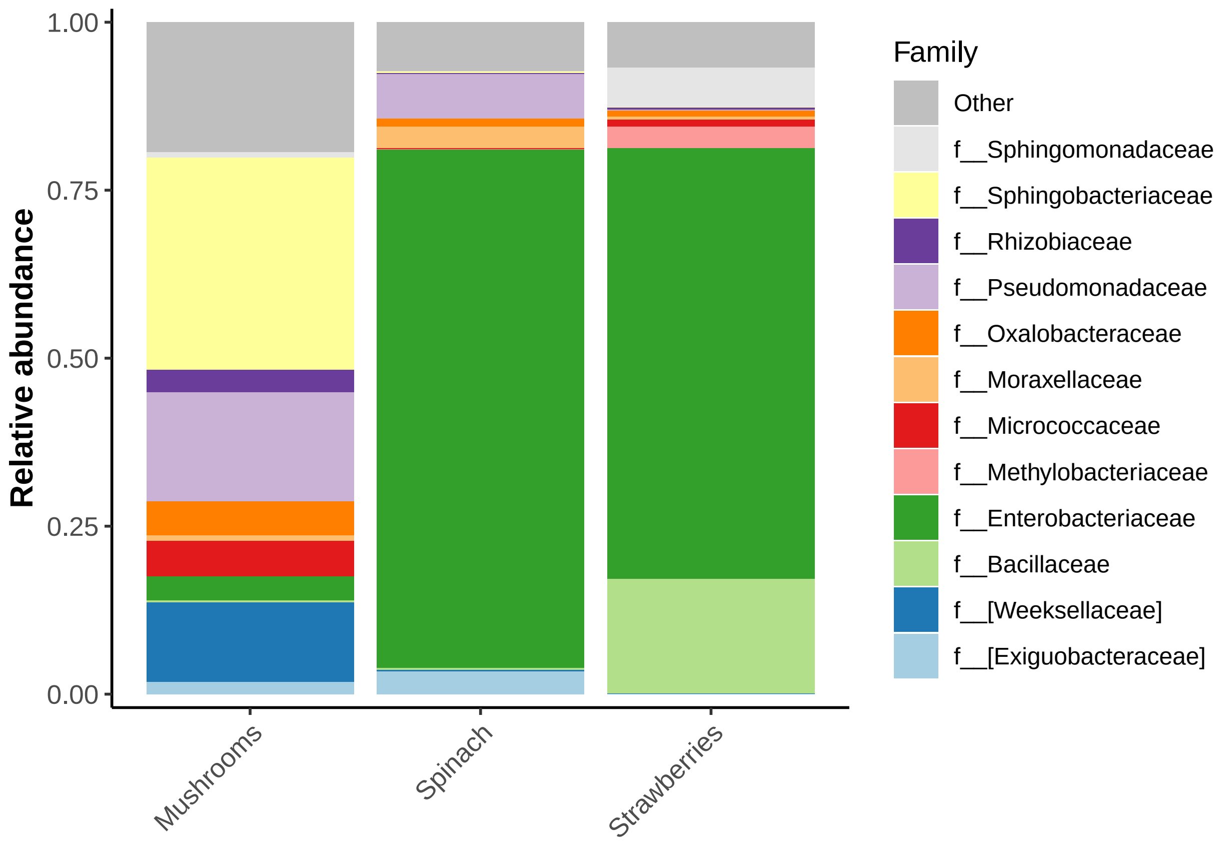
```
cliffplot_taxa_bars(input = input_filt_rar, level = 4, variable = "Sample_type")
```

```
## Warning: There were 3 warnings in `mutate()`.
## The first warning was:
## i In argument: `taxon = fct_relevel(taxon, "Unclassified", after = Inf)`.
## i In group 1: `group_by = Mushrooms`.
## Caused by warning:
## ! 1 unknown level in `f`: Unclassified
## i Run `dplyr::last_dplyr_warnings()` to see the 2 remaining warnings.
```



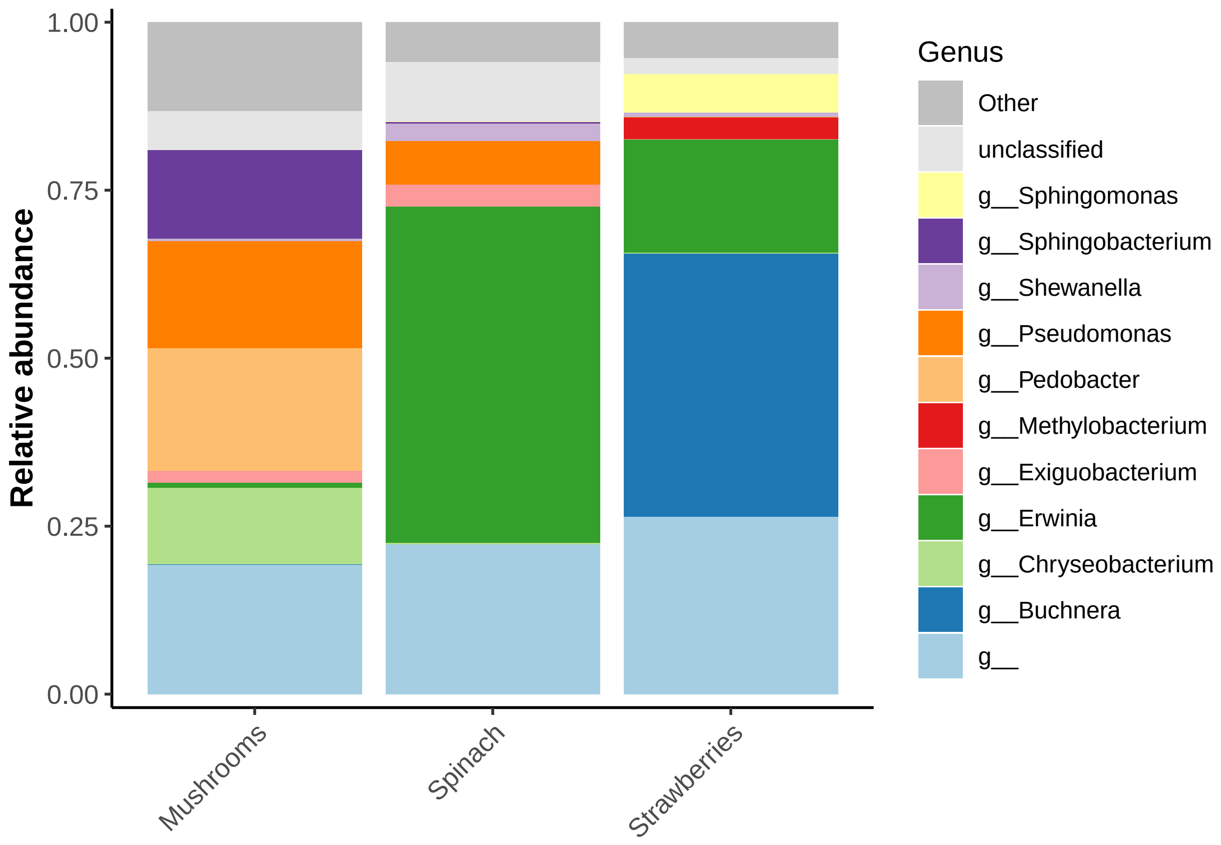
```
cliffplot_taxa_bars(input = input_filt_rar, level = 5, variable = "Sample_type")
```

```
## Warning: There were 3 warnings in `mutate()`.
## The first warning was:
## i In argument: `taxon = fct_relevel(taxon, "Unclassified", after = Inf)`.
## i In group 1: `group_by = Mushrooms`.
## Caused by warning:
## ! 1 unknown level in `f`: Unclassified
## i Run `dplyr::last_dplyr_warnings()` to see the 2 remaining warnings.
```



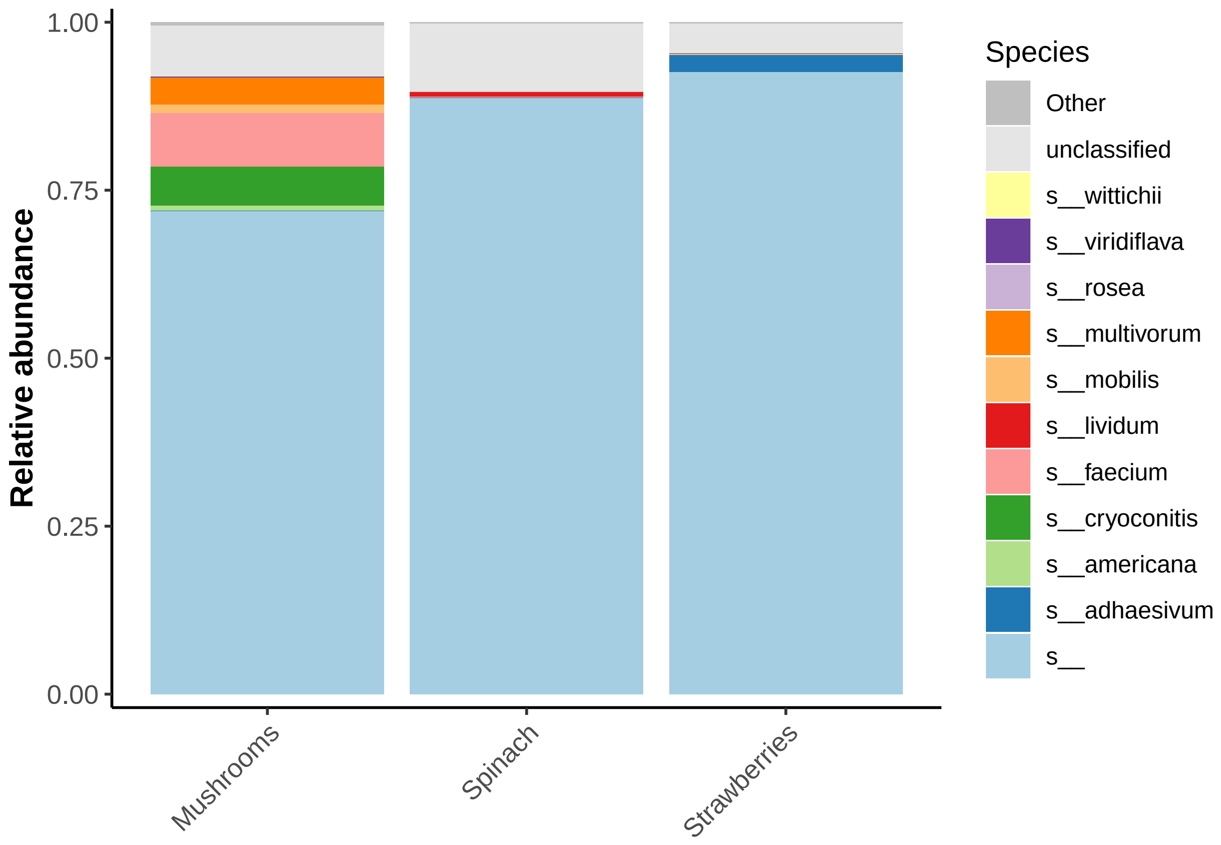
```r
cliffplot_taxa_bars(input = input_filt_rar, level = 6, variable = "Sample_type")
```

```
## Warning: There were 3 warnings in `mutate()`.
## The first warning was:
## i In argument: `taxon = fct_relevel(taxon, "Unclassified", after = Inf)`.
## i In group 1: `group_by = Mushrooms`.
## Caused by warning:
## ! 1 unknown level in `f`: Unclassified
## i Run `dplyr::last_dplyr_warnings()` to see the 2 remaining warnings.
```



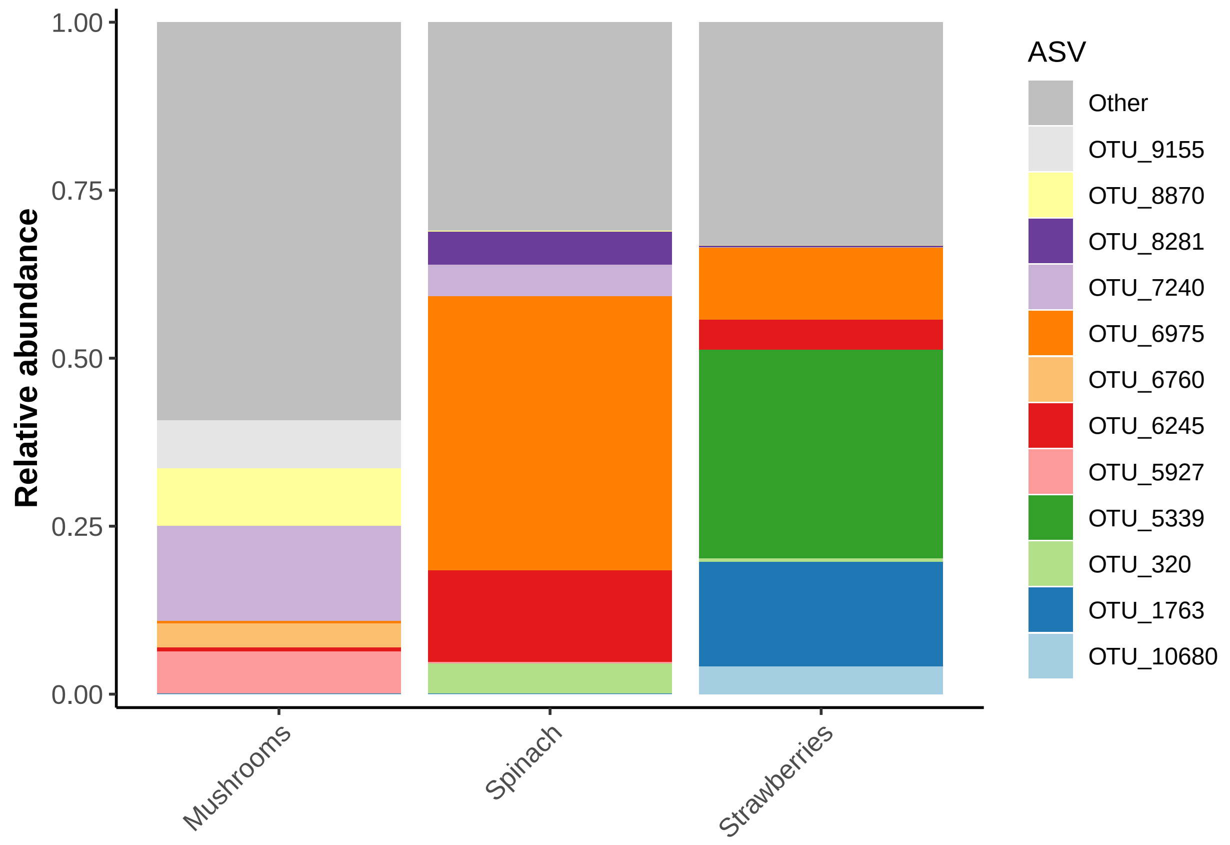
```r
cliffplot_taxa_bars(input = input_filt_rar, level = 7, variable = "Sample_type")
```

```
## Warning: There were 3 warnings in `mutate()`.
## The first warning was:
## i In argument: `taxon = fct_relevel(taxon, "Unclassified", after = Inf)`.
## i In group 1: `group_by = Mushrooms`.
## Caused by warning:
## ! 1 unknown level in `f`: Unclassified
## i Run `dplyr::last_dplyr_warnings()` to see the 2 remaining warnings.
```

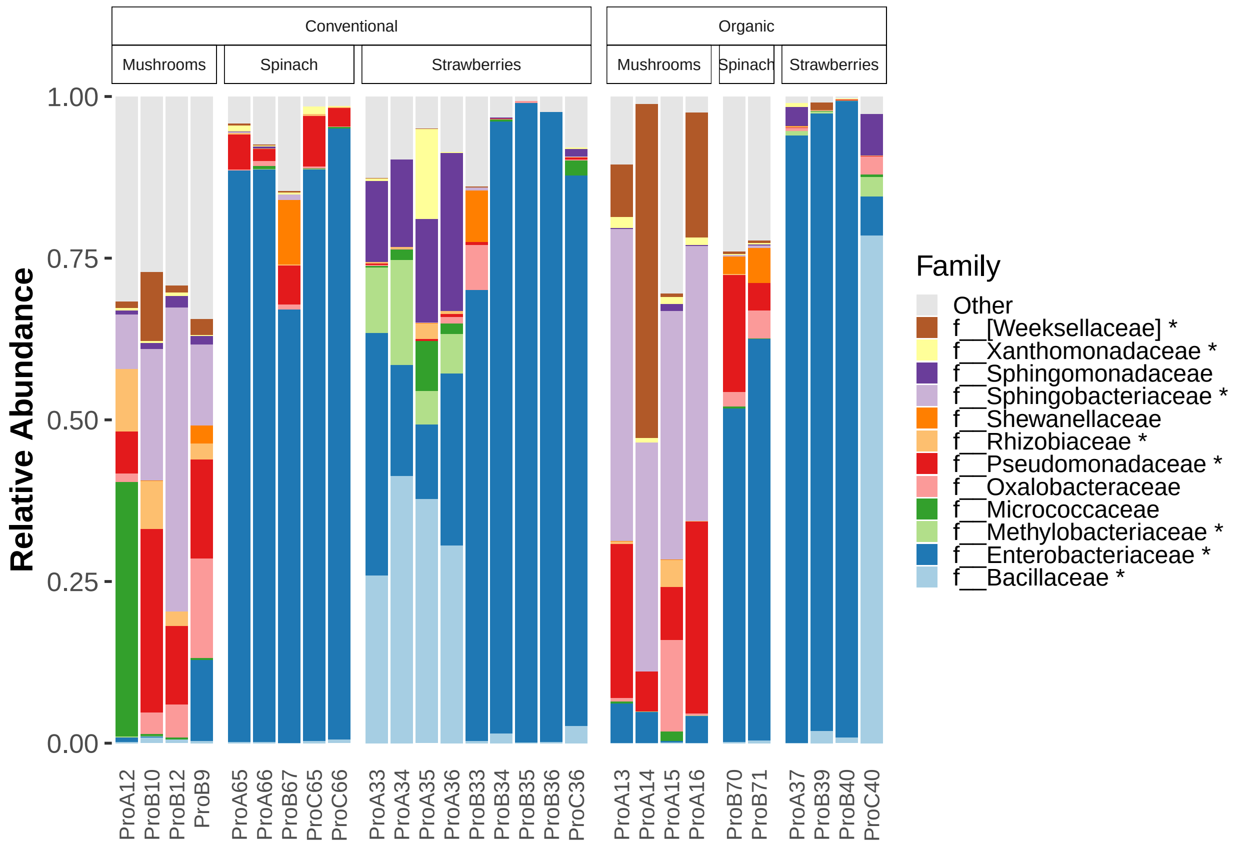


```
cliffplot_taxa_bars(input = input_filt_rar, level = 8, variable = "Sample_type")
```

```
## Warning: There were 3 warnings in `mutate()`.
## The first warning was:
## i In argument: `taxon = fct_relevel(taxon, "Unclassified", after = Inf)`.
## i In group 1: `group_by = Mushrooms`.
## Caused by warning:
## ! 1 unknown level in `f`: Unclassified
## i Run `dplyr::last_dplyr_warnings()` to see the 2 remaining warnings.
```



```r
# Add even more customization. Try a nested facet! These are great for parsing your taxonomy plot by di

# Make sampleID column
input_filt_rar$map_loaded$sampleID <- rownames(input_filt_rar$map_loaded)

# Summarize taxonomy. Let's do families.
tax_sum_families <- summarize_taxonomy(input_filt_rar, level = 5, report_higher_tax = FALSE)

# Get the plotting data. We'll also join the rest of map_loaded.
bars <- plot_taxa_bars(tax_sum_families,
                       input_filt_rar$map_loaded,
                       "sampleID",
                       num_taxa = 12,
                       data_only = TRUE) %>%
  mutate(taxon = fct_rev(taxon)) %>%
  left_join(., input_filt_rar$map_loaded, by = c("group_by" = "sampleID"))

# Run stats and add an asterisk to their name in the legend if significantly affected by treatment
fam_stats <- taxa_summary_by_sample_type(tax_sum_families,
                                         input_filt_rar$map_loaded,
                                         type_header = 'Sample_type',
                                         filter_level = 0.01,
                                         test_type = 'KW') %>%
  filter(rownames(.) %in% bars$taxon) %>%
  arrange(desc(rownames(.))) %>%
  mutate(Sig = ifelse(pvalsFDR < 0.05, "Pfdr < 0.05", "Pfdr > 0.05")) %>%
  mutate(Star = ifelse(pvalsFDR < 0.05, "*", "")) %>%
  rownames_to_column(var = "taxon") %>%
  arrange(match(taxon, levels(bars$taxon))) %>%
  mutate(StarLab = paste(taxon, Star, sep = " "))

# Plot
ggplot(bars, aes(group_by, mean_value, fill = taxon)) +
  geom_bar(stat = "identity", linewidth = 0.25) +
  labs(x = "Sample Type", y = "Relative Abundance", fill = "Family") +
  scale_fill_manual(values = c("grey90", brewer.pal(12, "Paired")[12:1]),
                    labels = c("Other", fam_stats$StarLab)) +
  scale_y_continuous(expand = c(0.01, 0.01)) +
  facet_nested(~ Farm_type + Sample_type, space = "free", scales = "free_x") +
  theme_classic() +
  theme(axis.title.y = element_text(face = "bold", size = 12),
        axis.title.x = element_blank(),
        axis.text.y = element_text(size = 10),
        axis.text.x = element_text(size = 8, angle = 90, hjust = 1, vjust = 0.5),
        axis.ticks.x = element_blank(),
        axis.line.x = element_blank(),
        strip.text = element_text(size = 6),
        strip.background = element_rect(linewidth = 0.2),
        axis.line.y = element_blank(),
        legend.margin = margin(0, 0, 0, 0, unit = "pt"),
        legend.box.margin = margin(0, 0, 0, 0, unit = "pt"),
        legend.key.size = unit(0.3, "cm"),
        panel.spacing.x = unit(c(0.2, 0.2, 0.4, 0.2, 0.2), "lines"))
```



```
# Test (run a Kruskal-Wallis test on all families with mean rel abund. > filter_level in at least one o
taxa_summary_by_sample_type(tax_sum_families,
                            input_filt_rar$map_loaded,
                            type_header = 'Sample_type',
                            filter_level = 0.05,
                            test_type = 'KW')
```

```
##                                pvals     pvalsBon     pvalsFDR   Mushrooms
## f__Pseudomonadaceae    1.462593e-05 0.0001170074 0.0001170074 0.162671233
## f__Sphingobacteriaceae 5.463909e-05 0.0004371127 0.0002185564 0.316122234
## f__[Weeksellaceae]     1.246078e-04 0.0009968628 0.0003322876 0.118545838
## f__Enterobacteriaceae  4.669735e-04 0.0037357879 0.0009339470 0.035958904
## f__Bacillaceae         1.761396e-02 0.1409117049 0.0281823410 0.002502634
## f__Oxalobacteraceae    6.710803e-02 0.5368642596 0.0894773766 0.050316122
## f__Sphingomonadaceae   7.023547e-02 0.5618837856 0.0802691122 0.007639621
## f__Micrococcaceae      5.776766e-01 4.6214126447 0.5776765806 0.052555321
##                             Spinach Strawberries
## f__Pseudomonadaceae    0.066084600 0.0014590257
## f__Sphingobacteriaceae 0.002258016 0.0004863419
## f__[Weeksellaceae]     0.001806413 0.0012158547
## f__Enterobacteriaceae  0.771940388 0.6403501662
## f__Bacillaceae         0.002709619 0.1703817784
## f__Oxalobacteraceae    0.012494355 0.0094026100
## f__Sphingomonadaceae   0.000752672 0.0594147686
## f__Micrococcaceae      0.001806413 0.0108616357
```

```
# Or Wilcoxon for 2 levels (farm type)
taxa_summary_by_sample_type(tax_sum_families,
                            input_filt_rar$map_loaded,
                            type_header = 'Farm_type',
                            filter_level = 0.05,
                            test_type = 'MW')
```

```
## Warning in wilcox.test.default(x = DATA[[1L]], y = DATA[[2L]], ...): cannot
## compute exact p-value with ties

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## compute exact p-value with ties
```

```
##                             pvals  pvalsBon  pvalsFDR Conventional    Organic
## f__[Weeksellaceae]     0.1246744 0.6233719 0.6233719  0.009073879 0.08134879
## f__Bacillaceae         0.1410588 0.7052939 0.3526469  0.079674511 0.08198103
## f__Sphingobacteriaceae 0.3120693 1.5603463 0.5201154  0.049994146 0.16512118
## f__Enterobacteriaceae  0.5330298 2.6651492 0.6662873  0.543730242 0.42286617
## f__Pseudomonadaceae    0.6118674 3.0593371 0.6118674  0.048823323 0.09041096
```