Scripts: Bacterial communities in the rhizosphere at different growth stages of maize cultivated in soil under conventional and conservation agricultural practices

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I. QIIME2 AND PICRUST2 SCRIPTS

Raw sequences were import to QIIME2 (Bolyen et al. 2019) workflow and then PICRUST2 (Douglas et al. 2020) was done to predict funcionality.

IMPORT TO QIIME AND DEMULTIPLEX SEQUENCES

```
qiime tools import --type EMPPairedEndSequences \
--input-path barcode_extracted/ \
--output-path yen.qza
```

–type : type of file , in this case paired end sequences. Check other import types[1].

–input-path: directory with the files to import

–output-path: artifact name output

And then, we perform the demultiplexing:

```
qiime demux emp-paired  \
--i-seqs yen.qza \
--m-barcodes-file Map_rhizos.txt \
--m-barcodes-column BarcodeSequence \
--o-per-sample-sequences demux.qza \
--o-error-correction-details errordetails.qza \
--p-no-golay-error-correction
```

–i-seqs : artifact with the import paired end sequences

–m-barcodes-file : mapping file containing information of the sequences

–m-barcodes-column: column name of the Barcode sequences

–o-per-sample-sequences : output of the sequences demultiplexed

–o-error-correction-details: file with correction details

–p-no-golay-error-correction: by default perform a correction with a barcode of 12 nt if not use this option (in our case is 16 nt)

To visualice the demux file:

[1] https://docs.qiime2.org/2021.4/tutorials/importing/

```
qiime demux summarize
--i-data demux.qza \
--o-visualization  demux.qzv
```

–i-data : demultiplexed and/or trimmed sequences

–o-visualization : output

In this case, due to de the low quality of reverse reads we will continue with the forward sequences and let's set the truncation length of 120 bp for forward reads.

RUN DADA2

```
qiime dada2 denoise-single \
--i-demultiplexed-seqs ../demultiplex/demux_yen.qza \
--p-trim-left 0 --p-trunc-len 120 \
--o-representative-sequences rep-seq-dada-forward.qza \
--o-table table-dada-forward.qza \
--o-denoising-stats stats-dada-forward.qza
```

–i-demultiplexed-seqs : demultiplexed and trimmed sequences

-p-trunc-len-f : length to trunc in forward sequences sequences to obtain good quality (usually when sequencing drops)

-p-trunc-len-r : length to trunc in resverse sequences sequences to obtain good quality (usually when sequencing drops)

–output-dir : output directory that will contain feature-table and representative sequences

FILTERING FORM ALIGNMENT (REMOVE UNASSINGED BASED ON GREEN GENES DATABASE)

First, we do the alignment against the green genes database:

```
qiime quality-control exclude-seqs \
--i-query-sequences rep-seq-dada-forward.qza \
--i-reference-sequences ../references/99_otus.qza \
--p-method vsearch \
--p-perc-identity 0.97 \
--p-perc-query-aligned 0.95 \
--p-threads 4 \
--o-sequence-hits hits.qza \
--o-sequence-misses misses.qza
```

–i-query-sequences : representative sequences obtained from dada2

–i-reference-sequences : reference sequences imported to qiime2

–p-method : alignment method

–p-perc-identity : identity percent

–p-perc-query-aligned : query aligned percent

–p-threads : number of threads

–o-sequence-hits : output with hits sequences

–o-sequence-misses : output with misses sequences (not aligned)

Now, filter the feature table to remove this misses sequences:

```
qiime feature-table filter-features \
--i-table table-dada-forward.qza \
--m-metadata-file misses.qza \
--o-filtered-table no-misses-table.qza  \
--p-exclude-ids
```

–i-table : feature table from dada2

–m-metadata-file : metadata mapping file

–o-filtered-table : filtered table

–p-exclude-ids : argument to exclude the ids from 'misses' file

ASSIGN TAXONOMY

```
qiime feature-classifier classify-sklearn \
--i-reads rep-seq-dada_forward.qza \
--i-classifier /home/steph/Descargas/gg-13-8-99-nb-classifier.qza \
--o-classification taxonomy.qza
```

cclassify-sklearn : using sklearn (other options are vsearch and blast)

–i-reads : seqs merged

–i-classifier: artifact classifier full-length (https://docs.qiime2.org/2021.4/data-resources/)

–o-classification output artifact with taxonomy

FILTERING TABLE

- **Removing taxa of chloroplast and mitochondria**

```
qiime taxa filter-table
--i-table no-misses-table.qza
--i-taxonomy taxonomy.qza
--p-exclude mitochondria,chloroplast
--o-filtered-table table_filtered.qza
```

–i-table : merge table

–i-taxonomy : taxonomy (from assign taxonomy)

–p-exclude : taxa to exclude

–o-filtered-table : output/artifact

- **Visualizing the taxonomy in a barplot**

```
qiime taxa barplot --i-table table_filtered.qza \
--i-taxonomy taxonomy.qza \
--m-metadata-file Map_rhizos.txt \
--o-visualization taxa_barplot.qzv


qiime tools view taxa-barplot.qzv
```

–i-table : input table

–m-metadata-file : mapping file

–i-taxonomy : taxonomy

–o-visualization: .qzv of barplot

FILTERING SEQUENCES

For this step we will filter the representative sequences base on the table filtered.

```
qiime feature-table filter-seqs \
--i-data rep-seq-dada-forward.qza \
--i-table table_filtered.qza \
--o-filtered-data rep-seqs-filter-exclude.qza
```

–i-data : input sequences

–i-table : input table use to filter

–o-filtered-data : output/filtered sequences

BUILDING THE TREE

For this step we will build the phylogenetic tree *denovo*.

```
qiime phylogeny align-to-tree-mafft-fasttree \
--i-sequences rep-seqs-filter-exclude.qza \
-output-dir tree/
```

–i-sequences : sequences filtered

–output-dir : output director that will contain the alignment, masked alignment, the tree and the rooted treed.

EXPORTING SEQUENCES, TABLE AND TAXONOMY

```
#export sequences
qiime tools export \
--input-path rep-seqs-filter-exclude.qza \
--output-path exported

#expor the feature table
qiime tools export \
--input-path .table_filtered.qza \
--output-path exported/

#export the taxonomy
qiime tools export \
--input-path taxonomy.qza \
--output-path exported/

#join the feature table and taxonomy
biom add-metadata \
-i exported/feature-table_grouped.biom \
--observation-metadata-fp  exported/taxonomy.tsv \
-o otutable_with_taxonomy.biom

#convert biom to tsv to check the otutable (feature-table)
biom convert -i otutable_with_taxonomy.biom
-o otutable.txt --to-tsv --header-key taxonomy
```

–input-path: artifact to export (table or taxonomy)

–output-path: directory outpur

-i : feature-table in biom format

–observation-metadata-fp : taxonomy file (already changed)

-o: output

–to-tsv –header-key taxonomy : options to convert and add taxonomy to otutable/feature-table

PICRUST2

```
picrust2_pipeline.py \
-s exported/dna-sequences.fasta \
-i exported/feature-table.biom \
-o picrust2


add_descriptions.py \
-i picrust2/EC_metagenome_out/pred_metagenome_unstrat.tsv.gz \
-m EC -o picrust2/EC_metagenome_out/pred_metagenome_unstrat_descrip.tsv.gz

add_descriptions.py \
-i picrust2/pathways_out/path_abun_unstrat.tsv.gz \
-m METACYC -o picrust2/pathways_out/path_abun_unstrat_descrip.tsv.gz
```

-s : exported sequences from qiime2 in fasta format

-i : exported table from qiime2 in biom format

-o: directory that contains the results (EC, KO, pathways)

In the add_descriptions.py (script to add the descriptions to EC and pathways file):

-i : file output from PICRUST2 pipeline (EC or pathways)

-m METACYC/EC : map type

-o : output file with descriptions

The files obtained from these scripts were imported into R fro downstream analyses.

II. ALPHA AND FUNCTIONALITY PLOT

Loading libraries

```
library(hillR)
library(gplots)
library(lme4)
library(nlme)
library(ggplot2)
library(cowplot)
library(pgirmess) # includes PermTest()
library(dplyr)
```

Loading files and formatting

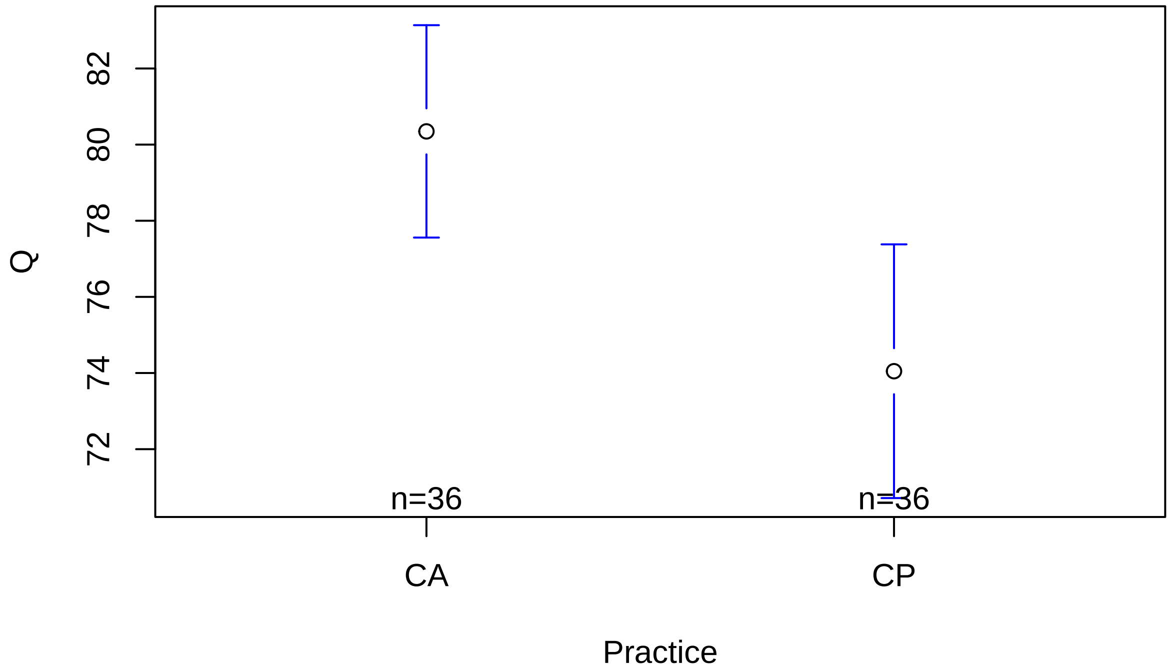
```
#Species as rows, traits as columns
EC_predicted <- read.delim(gzfile("../Data/EC_predicted.tsv.gz"), row.names=1)
KO_predicted <- read.delim(gzfile("../Data/KO_predicted.tsv.gz"), row.names=1)

#Sites as rows, species as columns
otutable <- read.delim("../Data/otutable_final_picrust2.txt", row.names=1)
otu_table<- otutable[,1:72]
totutable <- t(otu_table)
totutable <- totutable[ , match(rownames(KO_predicted), colnames(totutable))]
```

Functional diversity with Hill numbers

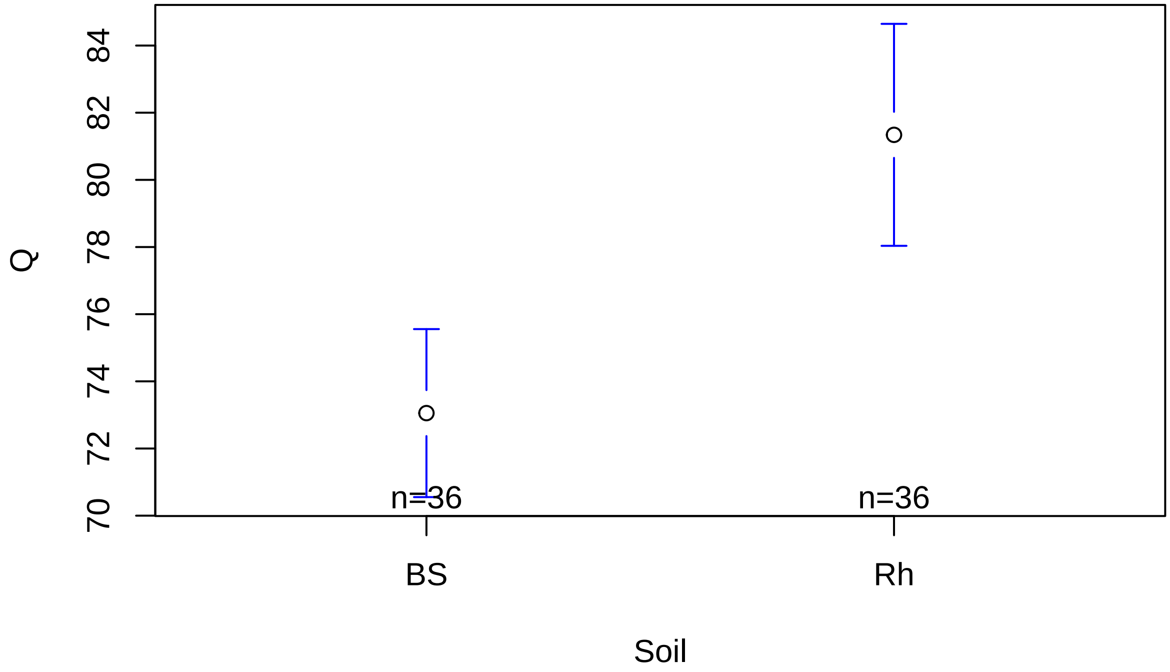
```
#Calculate the functional diversity (Not running due to long time)

#func_parti_q0<-hill_func_parti(totutable, traits = EC_predicted, q = 0)
#func_parti_q1<-hill_func_parti(totutable, traits = EC_predicted, q = 1)
#func_parti_q2<-hill_func_parti(totutable, traits = EC_predicted, q = 2)

#func_q0<- hill_func(totutable, traits = EC_predicted, q = 0)
#func_q1<- hill_func(totutable, traits = EC_predicted, q = 1)
#func_q2<- hill_func(totutable, traits = EC_predicted, q = 2)

#func_q0_KO<- hill_func(totutable, traits = KO_predicted, q = 0)
#func_q1_KO<- hill_func(totutable, traits = KO_predicted, q = 1)
#func_q2_KO<- hill_func(totutable, traits = KO_predicted, q = 2)

#write.table(t(func_q0), file="../Data/func_q0.txt", sep="\t")
#write.table(t(func_q2_KO), file="../Data/func_q2_KO.txt", sep="\t")
```

Plotting functional diversity

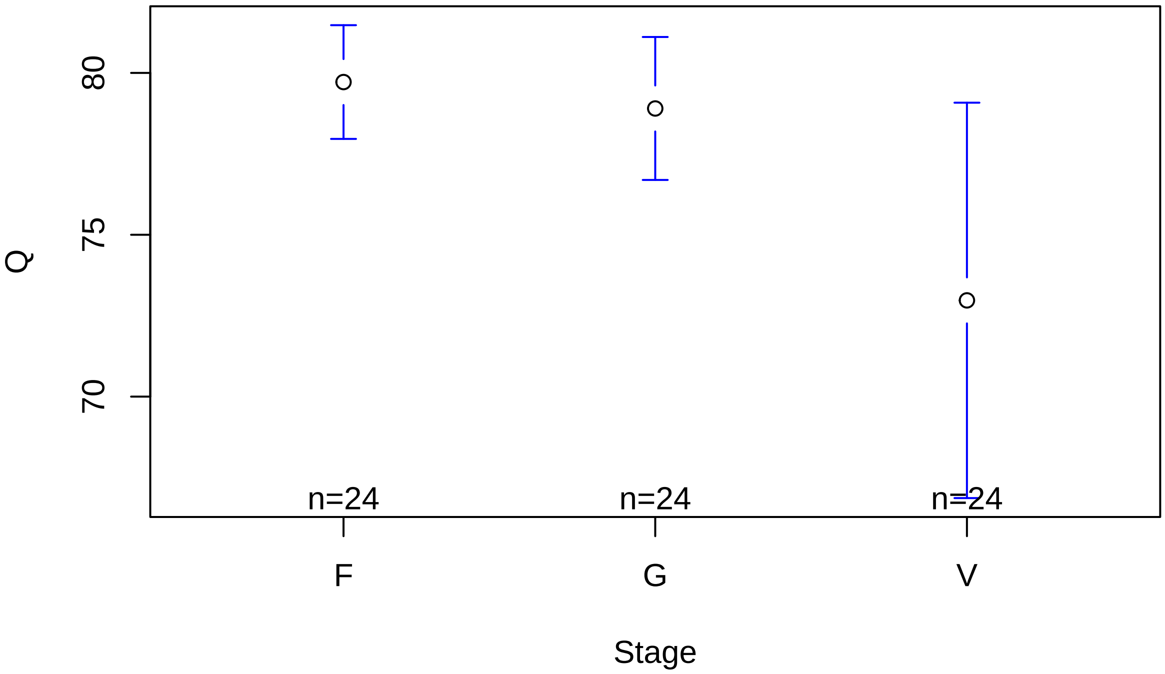
```
func_q0<- t(read.delim("../Data/func_q0.txt"))
func_q1<- t(read.delim("../Data/func_q1.txt"))
func_q2<- t(read.delim("../Data/func_q2.txt"))
Alpha.t_asv_table<- read.csv("../Data/Alpha-t_otu_table.txt.csv", check.names = F, row.names = 1)


funct_q0<-t(func_q0)
funct_q1<-t(func_q1)
funct_q2<-t(func_q2)

FD_q0<- merge(Alpha.t_asv_table, funct_q0, by=0)
FD_q1<- merge(Alpha.t_asv_table, funct_q1, by=0)
FD_q2<- merge(Alpha.t_asv_table, funct_q2, by=0)


plotmeans(Q~Practice, FD_q0, connect=F)
```



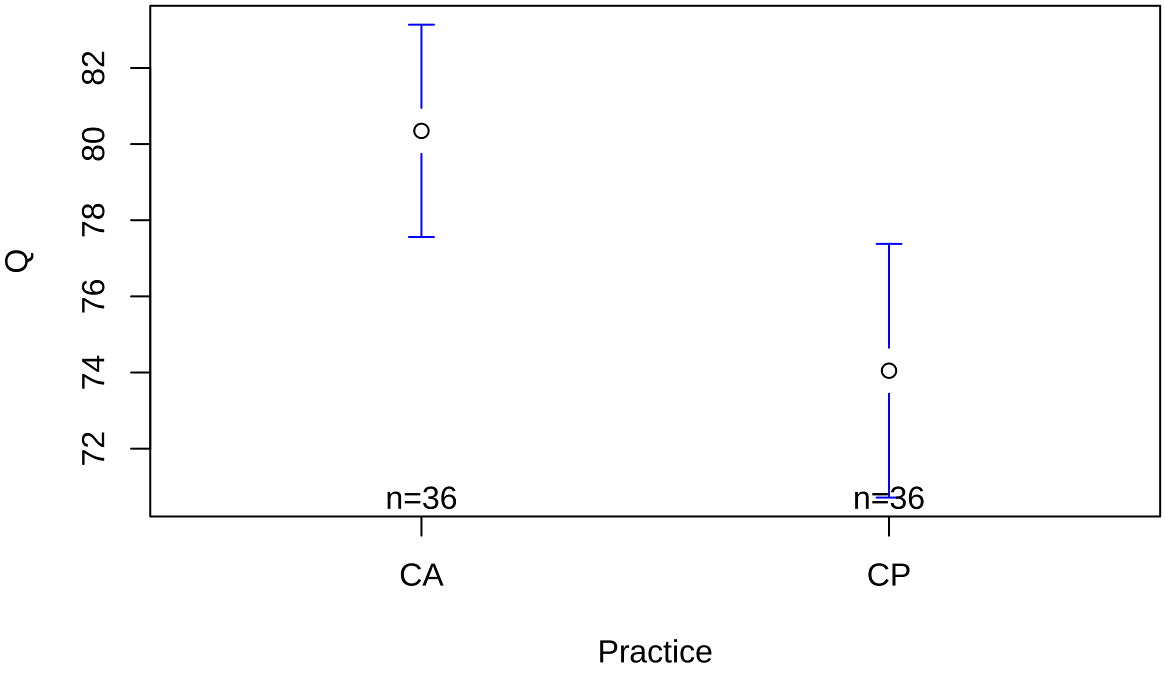
```
plotmeans(Q~Soil, FD_q0, connect=F)
```



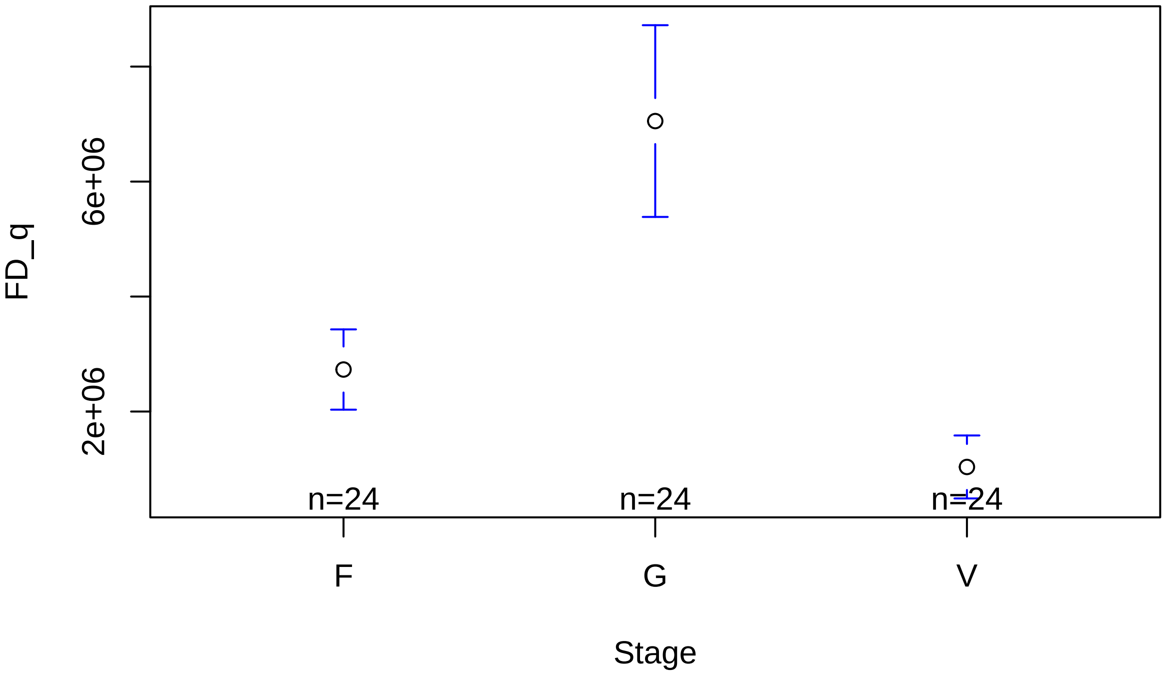
```
plotmeans(Q~Stage, FD_q1, connect=F)
```



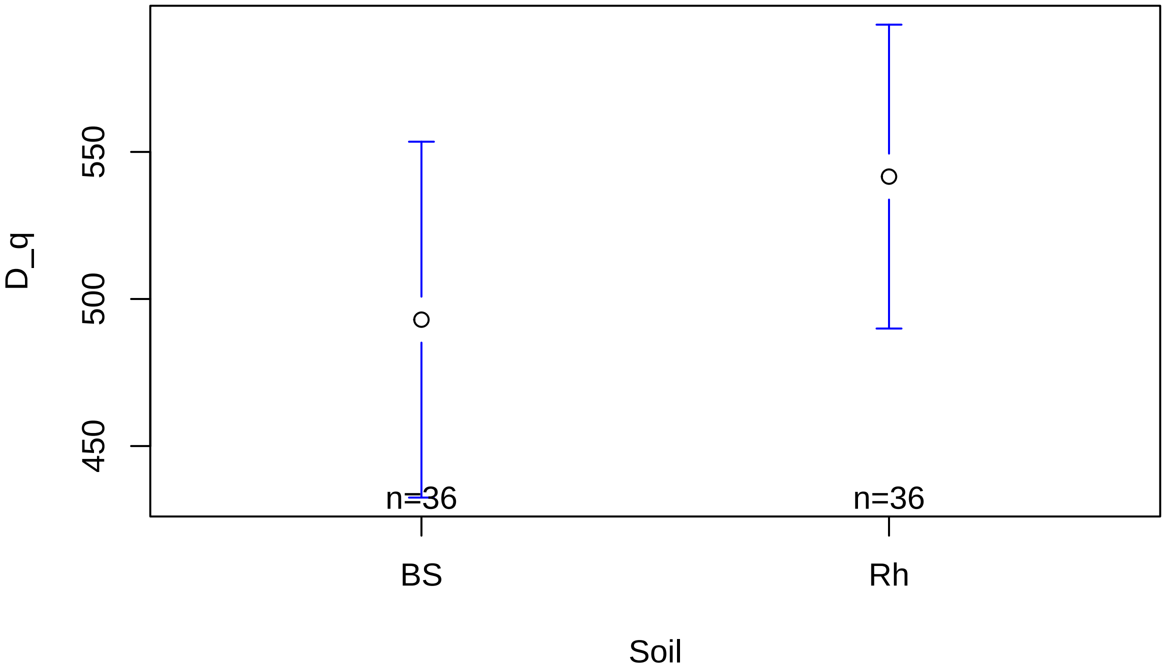
```
plotmeans(Q~Practice, FD_q0, connect=F)
```



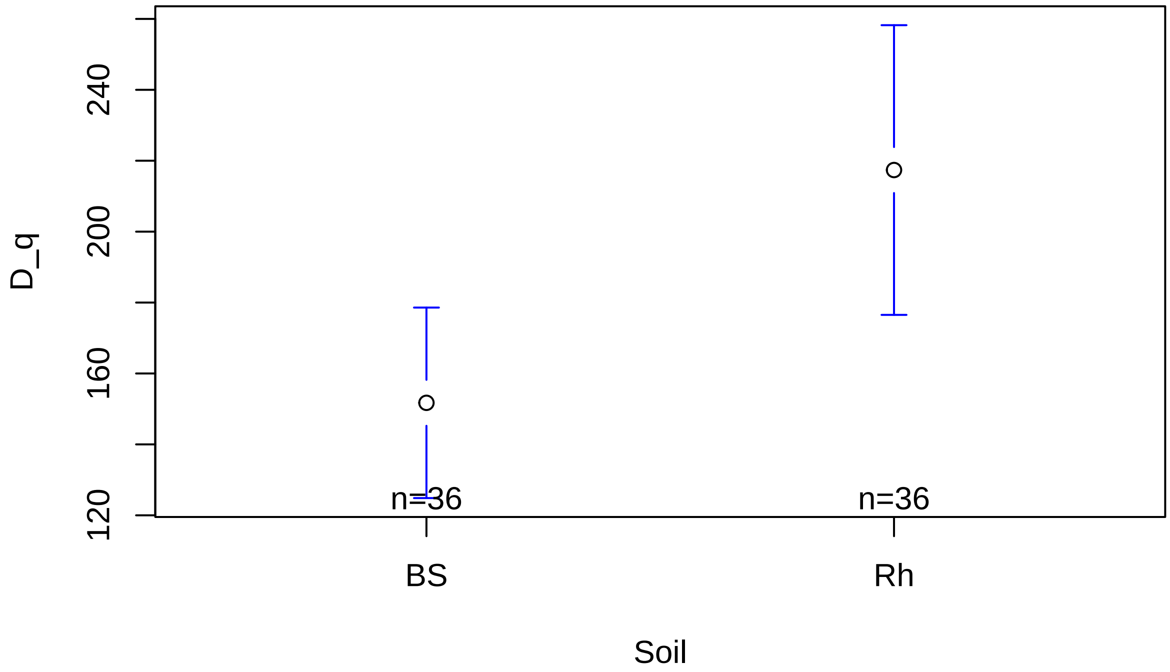
```
plotmeans(FD_q~Stage, FD_q1, connect=F)
```



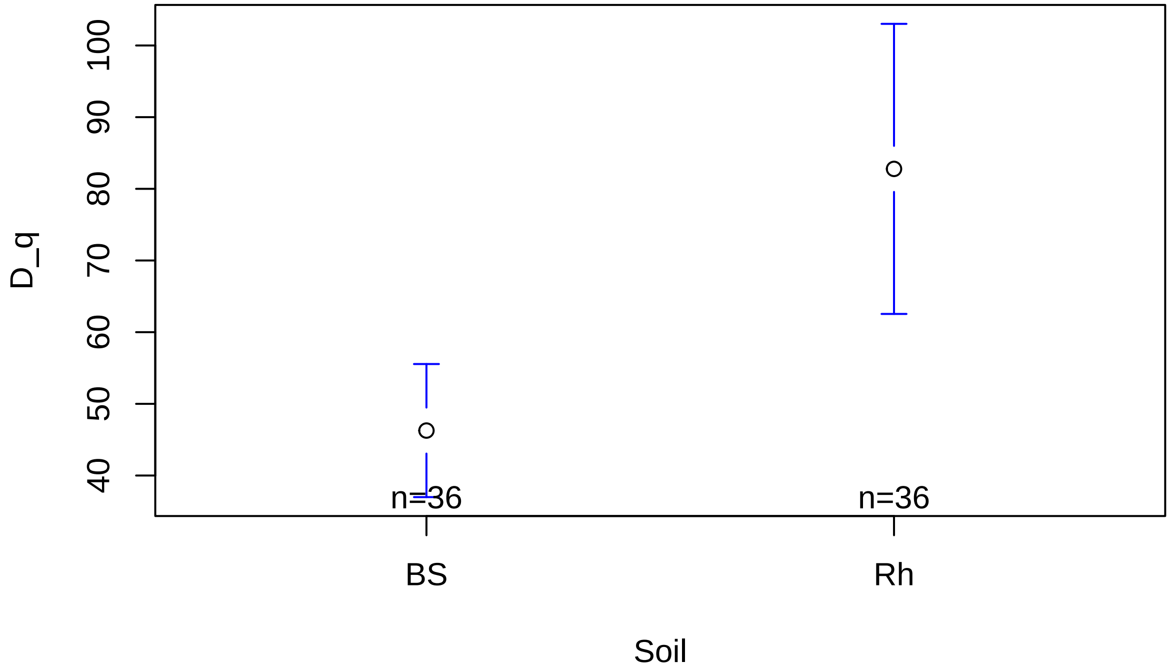
```
plotmeans(D_q~Soil, FD_q0, connect=F)
```



```
plotmeans(D_q~Soil, FD_q1, connect=F)
```



```
plotmeans(D_q~Soil, FD_q2, connect=F)
```



General linear model of functional diversity

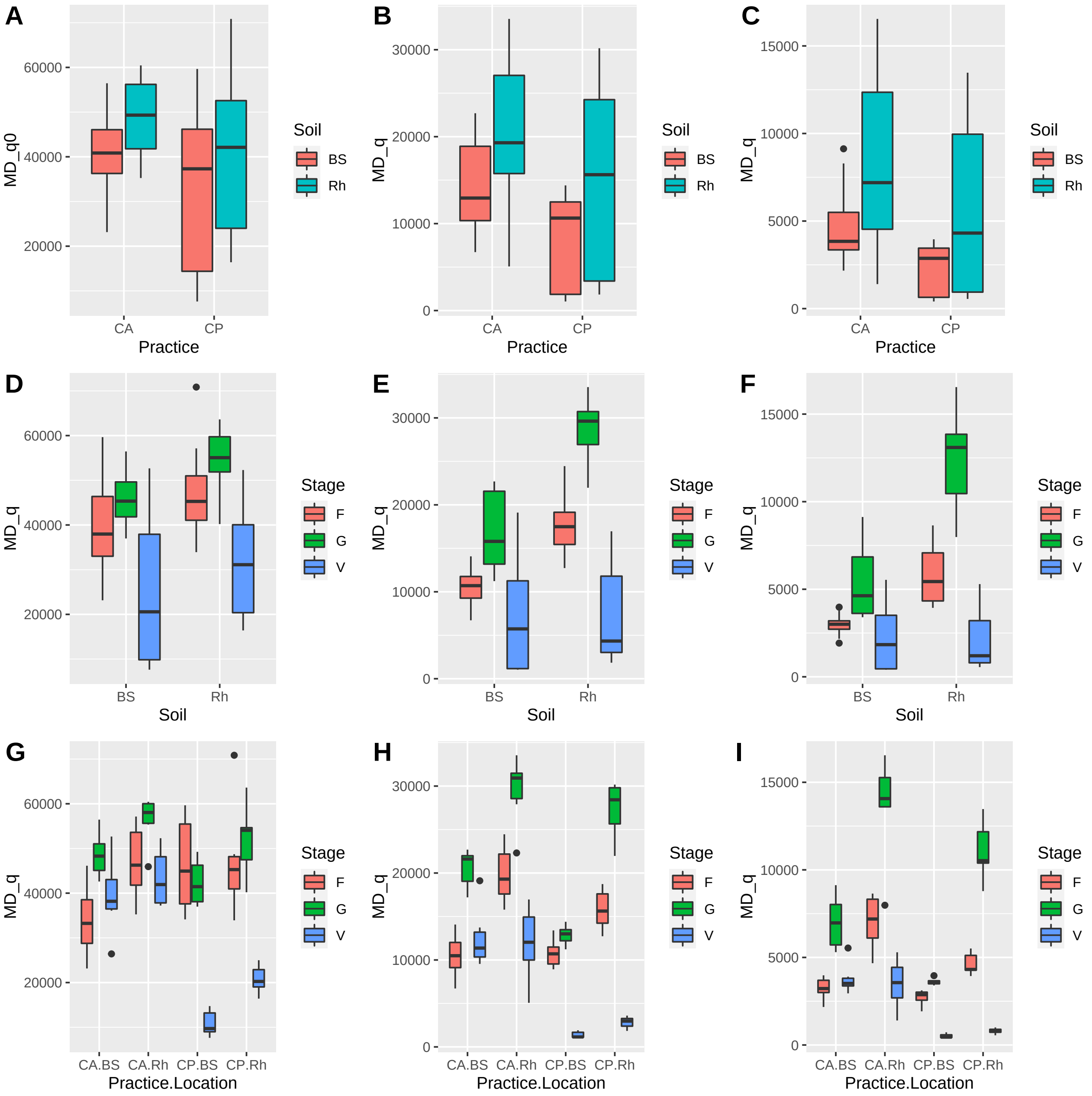
```
func_MDq<- read.delim("../Data/func_MDq.txt", check.names = F, row.names = 1)

a<-lme(FD_q~Practice.Location*Stage, random=~1 |Plot, FD_q2)%>%PermTest
summary(a)
```

```
##           Length Class      Mode
## resultats 1      data.frame list
## B          1      -none-     numeric
## call       2      -none-     call
```

```
b<-lme(FD_q~Soil, random=~1 |Plot, FD_q2)
summary(b)
```

```
## Linear mixed-effects model fit by REML
##   Data: FD_q2
##        AIC      BIC    logLik
##   2100.708 2109.702 -1046.354
##
## Random effects:
##  Formula: ~1 | Plot
##         (Intercept) Residual
## StdDev:    145905.9 704699.1
##
## Fixed effects:  FD_q ~ Soil
##                Value Std.Error DF  t-value p-value
## (Intercept) 216353.2  138262.8 67 1.564797  0.1223
## SoilRh      618085.1  166099.2 67 3.721181  0.0004
##  Correlation:
##        (Intr)
## SoilRh -0.601
##
## Standardized Within-Group Residuals:
##         Min          Q1         Med          Q3         Max
## -1.23073653 -0.63084391 -0.15614166  0.08395973  3.69128696
##
## Number of Observations: 72
## Number of Groups: 4
```

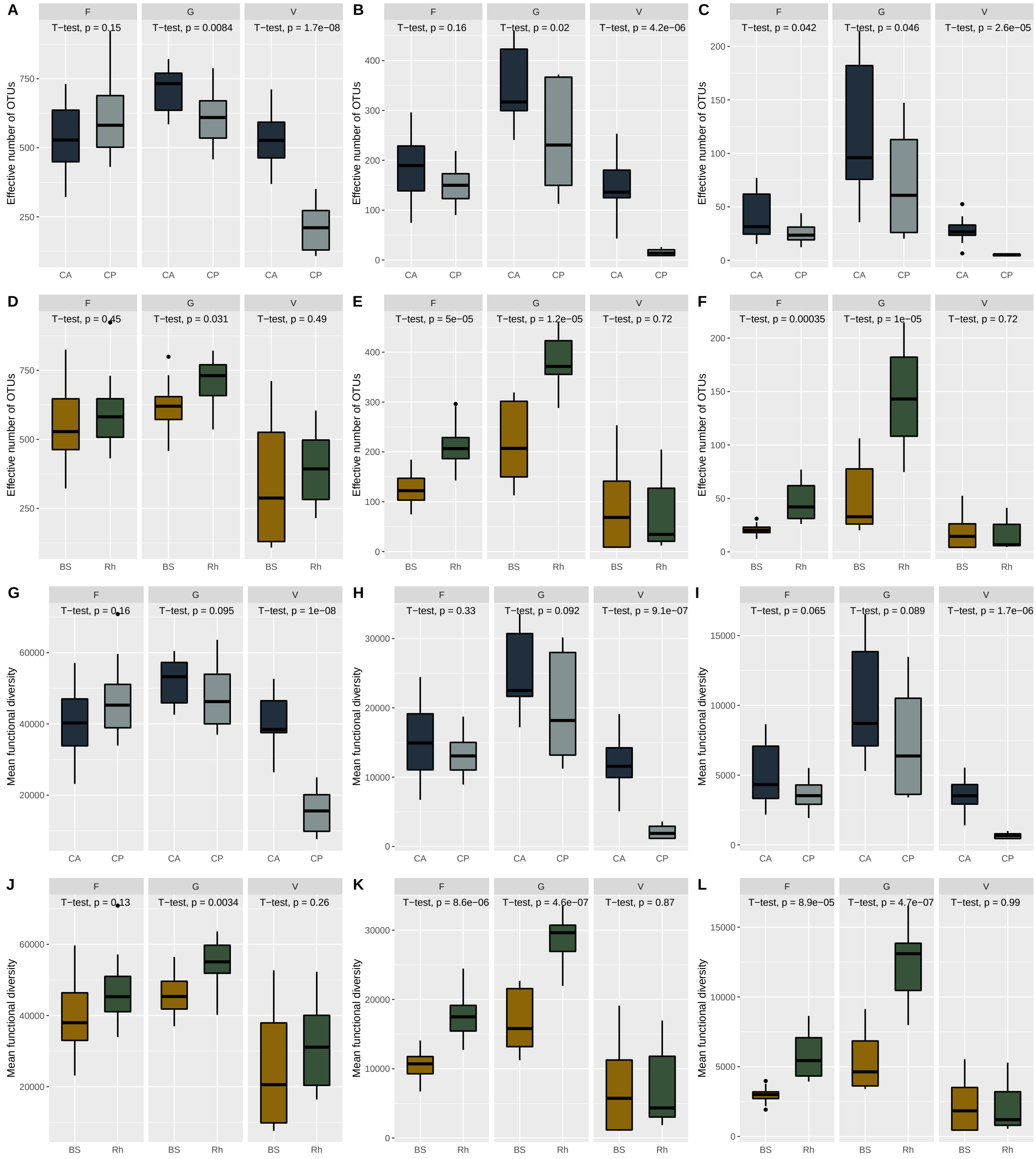
```
c<- lme(FD_q~Stage, random=~1 |Plot, FD_q2)%>%
PermTest


O<-ggplot(func_MDq, aes(x=Practice, y=MD_q0, fill=Soil))+
  geom_boxplot()


I<-ggplot(FD_q1, aes(x=Practice, y=MD_q, fill=Soil))+
  geom_boxplot()

II<- ggplot(FD_q2, aes(x=Practice, y=MD_q, fill=Soil))+
  geom_boxplot()

Os<-ggplot(FD_q0, aes(x=Soil, y=MD_q, fill=Stage))+
  geom_boxplot()

Is<-ggplot(FD_q1, aes(x=Soil, y=MD_q, fill=Stage))+
  geom_boxplot()

IIs<- ggplot(FD_q2, aes(x=Soil, y=MD_q, fill=Stage))+
  geom_boxplot()

Oss<-ggplot(FD_q0, aes(x=Practice.Location, y=MD_q, fill=Stage))+
  geom_boxplot()

Iss<-ggplot(FD_q1, aes(x=Practice.Location, y=MD_q, fill=Stage))+
  geom_boxplot()

IIss<- ggplot(FD_q2, aes(x=Practice.Location, y=MD_q, fill=Stage))+
  geom_boxplot()

r<-plot_grid(O, I, II, Os, Is, IIs, Oss, Iss, IIss,
          labels = "AUTO",
          label_size = 17, nrow=3, ncol = 3)
r
```



```
#pdf("FigX_FUNDIV-interactions.pdf", width=10, height=8)
#print(r)
#dev.off()
```

Plot S3

```
library(ggpubr)
library(cowplot)
func_MDq <- read.delim("../Data/func_MDq.txt", row.names=1)

F0.p <- ggboxplot(data = func_MDq, x = "Practice", y= "MD_q0",
                fill= "Practice", palette = c("#212F3D", "#839192"),
                width = 0.6, lwd=0.8, facet.by = "Stage")  +
    labs(x = element_blank(), y = "Mean functional diversity")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")
F1.p <- ggboxplot(data = func_MDq, x = "Practice", y= "MD_q1",
                  fill = "Practice", palette = c("#212F3D", "#839192"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Mean functional diversity")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")
F2.p <- ggboxplot(data = func_MDq, x = "Practice", y= "MD_q2",
                  fill = "Practice", palette = c("#212F3D", "#839192"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Mean functional diversity")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")

F0.s <- ggboxplot(data = func_MDq, x = "Soil", y= "MD_q0",
                  fill = "Soil", palette = c("darkgoldenrod4", "#365238"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Mean functional diversity")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")
F1.s <- ggboxplot(data = func_MDq, x = "Soil", y= "MD_q1",
                  fill = "Soil", palette = c("darkgoldenrod4", "#365238"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Mean functional diversity")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")
F2.s <- ggboxplot(data = func_MDq, x = "Soil", y= "MD_q2",
                  fill = "Soil", palette = c("darkgoldenrod4", "#365238"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Mean functional diversity")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")

div <- read.delim("../Data/Alpha-t_asv_table.txt", row.names=1)

D0.p <- ggboxplot(data = div, x = "Practice", y= "q0",
                  fill = "Practice", palette = c("#212F3D", "#839192"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Effective number of OTUs")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")
D1.p <- ggboxplot(data = div, x = "Practice", y= "q1",
                  fill = "Practice", palette = c("#212F3D", "#839192"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Effective number of OTUs")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")

D2.p <- ggboxplot(data = div, x = "Practice", y= "q2",
                  fill = "Practice", palette = c("#212F3D", "#839192"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Effective number of OTUs")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")

D0.s <- ggboxplot(data = div, x = "Soil", y= "q0",
                  fill = "Soil", palette = c("darkgoldenrod4", "#365238"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Effective number of OTUs")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")
D1.s <- ggboxplot(data = div, x = "Soil", y= "q1",
                  fill = "Soil", palette = c("darkgoldenrod4", "#365238"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Effective number of OTUs")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")

D2.s <- ggboxplot(data = div, x = "Soil", y= "q2",
                  fill = "Soil", palette = c("darkgoldenrod4", "#365238"),
                  width = 0.6, lwd=0.8, facet.by = "Stage")  +
  labs(x = element_blank(), y = "Effective number of OTUs")+
  theme_gray() +
  theme(text = element_text (size = 12))+
  theme(legend.position = "none")+
  theme(plot.title = element_text("q=0"))+
  theme(legend.position = "none",
        axis.ticks.x = element_blank())+
  stat_compare_means(method = "t.test")


r<-plot_grid(D0.p, D1.p,D2.p,D0.s,D1.s,D2.s, F0.p, F1.p,F2.p,F0.s,F1.s,F2.s,
             labels = "AUTO",
             label_size = 17, nrow=4, ncol = 3)
r
```



```
#pdf("FigS3_Div_block_by_stage.pdf", width=16, height=18)
#print(r)
#dev.off()
```

III. BETA-DIVERSITY PLOT

Loading libraries

```
library(cowplot)
library(tidyverse)
library(ggpubr)
library(circlize)
library(viridis)
library(RColorBrewer)
library(grid)
library(ggplot2)
```

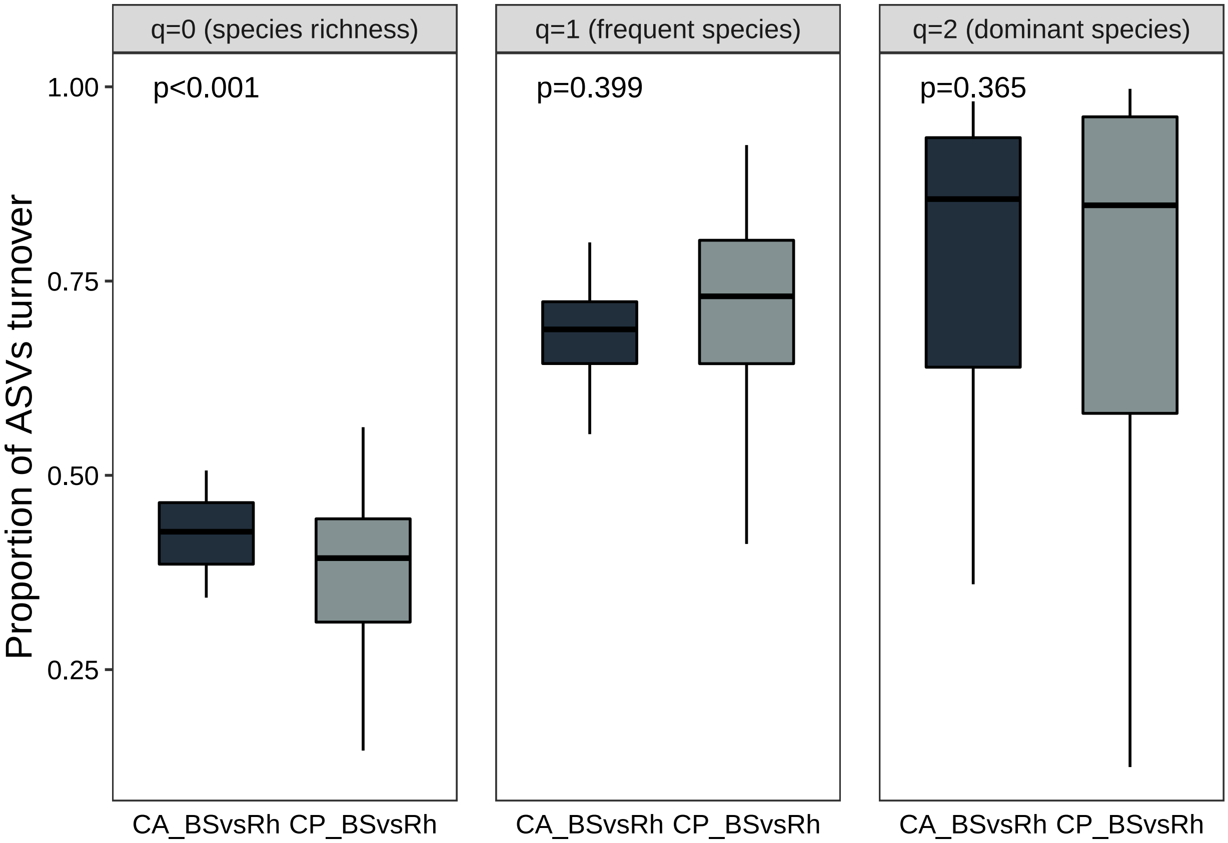
Loading and formatting files

```
beta<- read_tsv("../Data/beta_diversity.txt") %>% mutate(qs = case_when(
  q == 0  ~ "q=0 (species richness)",
  q == 1 ~ "q=1 (frequent species)",
  q == 2 ~ "q=2 (dominant species)")) %>% rename("ASVs_turnover" = TurnoverComp)
head(beta)
```

```
## # A tibble: 6 x 12
##       q ID1   ID2    beta LocalOverlap RegionalOverlap Homogeneity ASVs_turnover
##   <dbl> <chr> <chr> <dbl>        <dbl>           <dbl>       <dbl>         <dbl>
## 1     0 CAFB~ CAFR~  1.66        0.343           0.207       0.207         0.343
## 2     1 CAFB~ CAFR~  1.35        0.572           0.572       0.486         0.654
## 3     2 CAFB~ CAFR~  1.04        0.923           0.960       0.923         0.960
## 4     0 CAFB~ CAFR~  1.63        0.370           0.227       0.227         0.370
## 5     1 CAFB~ CAFR~  1.32        0.598           0.598       0.513         0.678
## 6     2 CAFB~ CAFR~  1.09        0.828           0.906       0.828         0.906
## # ... with 4 more variables: Comparison <chr>, PlotCompare <chr>, Type <chr>,
## #   qs <chr>
```

Treatment plot

```
ann_text_treatment<-data.frame(
  Comparison=c("CA_BSvsRh", "CA_BSvsRh", "CA_BSvsRh"),
  "ASVs_turnover"=c(1,1,1),
  qs=c("q=0 (species richness)","q=1 (frequent species)","q=2 (dominant species)"),
  label=c("p<0.001","p=0.399", "p=0.365")) #tittles and positiong in y axis

beta_treatment<- beta %>%
  filter(str_detect(Comparison, '^CA|^CP'))%>% ggplot(
    aes(y=`ASVs_turnover`,x=Comparison,  fill=Comparison)) +
  geom_boxplot(position=position_dodge(1), outlier.shape = NA, color="black",
             width=0.6)+theme_bw()+
  labs(y = "Proportion of ASVs turnover")+
  facet_grid(~qs, scales = "free")+
  theme(panel.spacing=unit(1,"lines"),
        # strip.background=element_rect(color="grey30", fill="gray90"),
        # panel.border=element_rect(color="black"),
        #strip.text.x = element_text(
        #  size = 12, color = "black", face = "bold"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        axis.title.x = element_blank(),
        legend.title = element_blank(),
        axis.title.y = element_text(size = 14),
        # legend.text = element_text(size=16),
        # axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        # legend.position = c(0.6,0.8),
        legend.direction = "vertical" ,
        legend.position = "none")+scale_fill_manual(values = c("#212F3D","#839192"))+
  geom_text(data = ann_text_treatment,label=ann_text_treatment$label)
beta_treatment
```

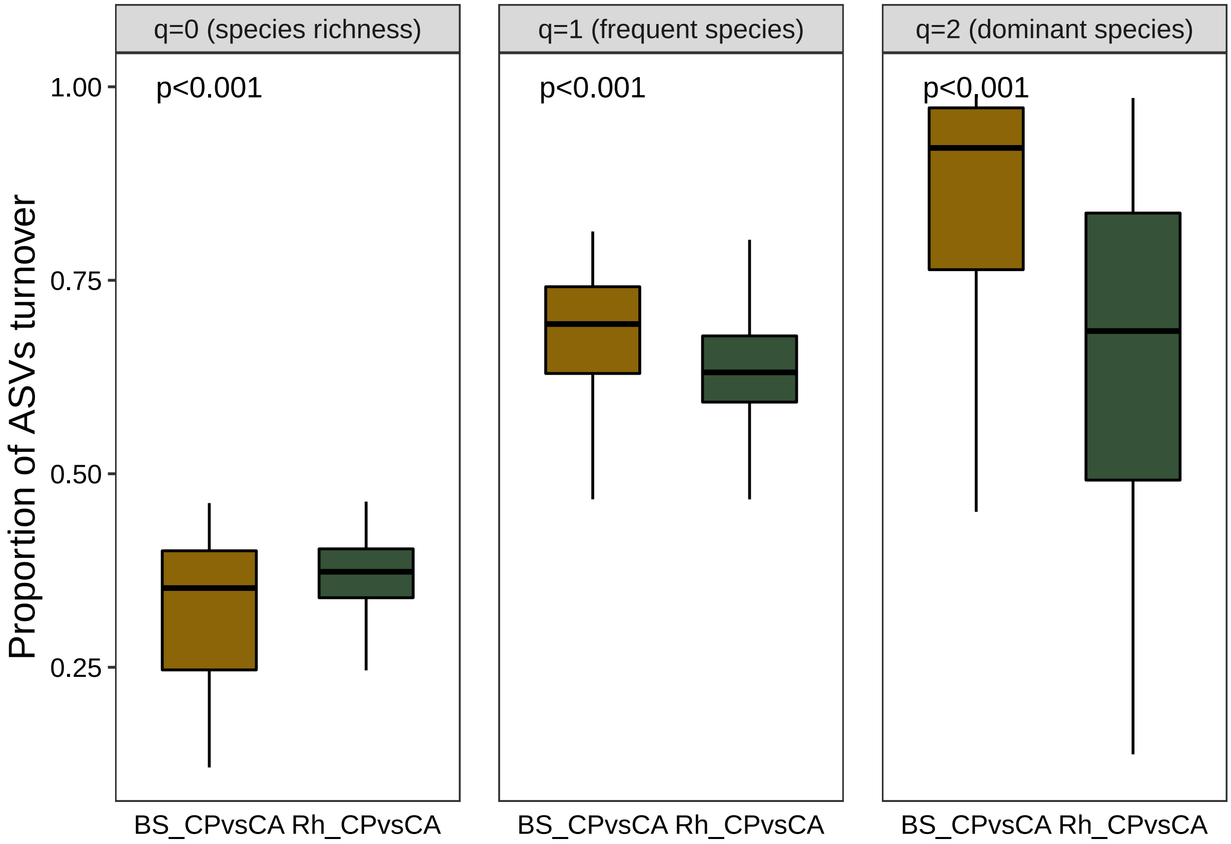


```
#pdf("fig_beta_treatment.pdf", width=6, height=3)
#print(beta_treatment)
#dev.off()
```

Soil Plot

```
ann_text_soil<-data.frame(
  Comparison=c("BS_CPvsCA", "BS_CPvsCA", "BS_CPvsCA"),
  "ASVs_turnover"=c(1,1,1),
  qs=c("q=0 (species richness)","q=1 (frequent species)","q=2 (dominant species)"),
  label=c("p<0.001","p<0.001", "p<0.001")) #tittles and positiong in y axis
```

```r
beta_soil<- beta %>%
  filter(!str_detect(Comparison, '^CA|^CP'))%>% ggplot(
    aes(y=`ASVs_turnover`,x=Comparison,  fill=Comparison)) +
  geom_boxplot(position=position_dodge(1), outlier.shape = NA, color="black",
               width=0.6)+theme_bw()+
  labs(y = "Proportion of ASVs turnover")+
  facet_grid(~qs, scales = "free")+
  theme(panel.spacing=unit(1,"lines"),
        # strip.background=element_rect(color="grey30", fill="gray90"),
        # panel.border=element_rect(color="black"),
        #strip.text.x = element_text(
        #  size = 12, color = "black", face = "bold"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        axis.title.x = element_blank(),
        legend.title = element_blank(),
        axis.title.y = element_text(size = 14),
        # legend.text = element_text(size=16),
        # axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        # legend.position = c(0.6,0.8),
        legend.direction = "vertical" ,
        legend.position = "none")+scale_fill_manual(values = c("darkgoldenrod4", "#365238"))+
  geom_text(data = ann_text_soil,label=ann_text_soil$label)
beta_soil
```



```
#pdf("fig_beta_soil.pdf", width=6, height=3)
#print(beta_soil)
#dev.off()
```

IV. SOIL FIGURE

Loading libraries

```
library(cowplot)
library(tidyverse)
library(ggpubr)
library(ComplexHeatmap)
library(circlize)
library(viridis)
library(RColorBrewer)
library(grid)
library(CoDaSeq)
library(ggplot2)
require(compositions) # exploratory d ata analysis of compositional data
require(zCompositions) # used for 0 substitution
require(ALDEx2) # used for per-OTU comparisons
library(CoDaSeq)
library(ggrepel)
```

Loadings files and Barplot Text annotations

```
alpha<- read.table("../Data/alpha_diversity") %>% gather(
  q0:q4, key = "q", value = "value") %>% filter(
    q %in% c("q0", "q1", "q2"))%>%mutate(qs= case_when(
  str_detect(q, "q0") ~ "q=0 (species richness)",
  str_detect(q, "q1") ~ "q=1 (frequent species)",
  str_detect(q, "q2") ~ "q=2 (dominant species)"))
head(alpha)
```

```
##   Practice Soil Practice.Location Stage Age Plant Plot ExpUnit  q value
## 1       CA   BS             CA.BS     F   1     1   18 CA.BS.F q0   426
## 2       CA   BS             CA.BS     F   1     2   18 CA.BS.F q0   646
## 3       CA   BS             CA.BS     F   1     3   18 CA.BS.F q0   510
## 4       CA   BS             CA.BS     F   1     1   59 CA.BS.F q0   546
## 5       CA   BS             CA.BS     F   1     2   59 CA.BS.F q0   391
## 6       CA   BS             CA.BS     F   1     3   59 CA.BS.F q0   322
##                       qs
## 1 q=0 (species richness)
## 2 q=0 (species richness)
## 3 q=0 (species richness)
## 4 q=0 (species richness)
## 5 q=0 (species richness)
## 6 q=0 (species richness)
```

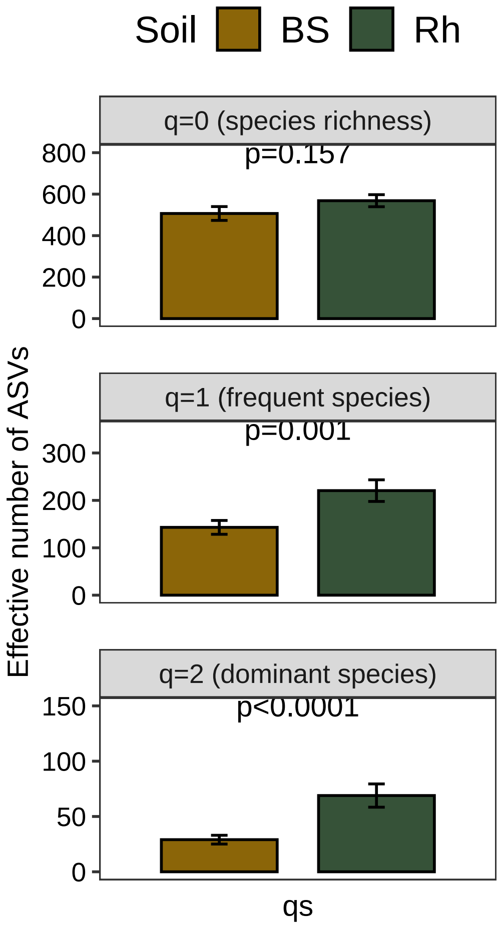
```
func<- read.table("../Data/func_MDq.txt") %>% gather(
  MD_q0:MD_q2, key = "q", value = "value")%>%mutate(fs= case_when(
  str_detect(q, "q0") ~ "q=0 (species richness)",
  str_detect(q, "q1") ~ "q=1 (frequent species)",
  str_detect(q, "q2") ~ "q=2 (dominant species)"))
head(func)
```

```
##   Practice Soil Practice.Location Stage Age Plant Plot ExpUnit     q    value
## 1       CA   BS             CA.BS     F   1     1   18 CA.BS.F MD_q0 29629.84
## 2       CA   BS             CA.BS     F   1     2   18 CA.BS.F MD_q0 46138.20
## 3       CA   BS             CA.BS     F   1     3   18 CA.BS.F MD_q0 36859.81
## 4       CA   BS             CA.BS     F   1     1   59 CA.BS.F MD_q0 39086.65
## 5       CA   BS             CA.BS     F   1     2   59 CA.BS.F MD_q0 28482.32
## 6       CA   BS             CA.BS     F   1     3   59 CA.BS.F MD_q0 23152.35
##                        fs
## 1 q=0 (species richness)
## 2 q=0 (species richness)
## 3 q=0 (species richness)
## 4 q=0 (species richness)
## 5 q=0 (species richness)
## 6 q=0 (species richness)
```

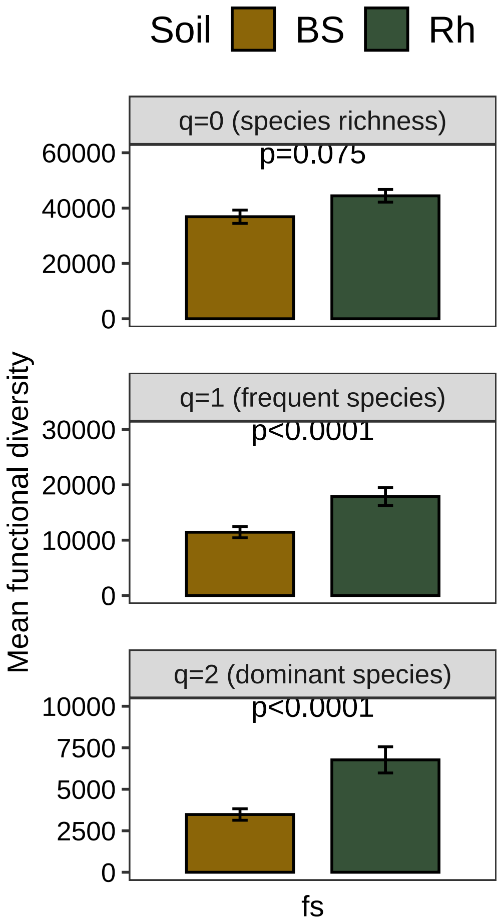
```
#df with the p values to show in the figures
ann_text<-data.frame(Soil=c("BS", "BS", "BS"),value=c(800,350,150),
qs=c("q=0 (species richness)","q=1 (frequent species)",
"q=2 (dominant species)"),label=c("p=0.157","p=0.001", "p<0.0001"))
#tittles and position in y axis


ann_text_f<-data.frame(Soil=c("BS", "BS", "BS"),value=c(60000,30000,10000),
fs=c("q=0 (species richness)","q=1 (frequent species)",
"q=2 (dominant species)"),label=c("p=0.075","p<0.0001", "p<0.0001"))
#tittles and position in y axis
```

Barplots alpha and functional diversity

```
#Alpha diversity barplot soil
boxplot_soil<-alpha %>%
  ggbarplot(x="qs", y="value", fill = "Soil", add = "mean_se",
            position = position_dodge())+
    theme_bw()+
  labs(y = "Effective number of ASVs")+
  facet_wrap(~qs, scales = "free", dir = "v")+
  theme(panel.spacing=unit(1,"lines"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        legend.title = element_text(size = 14),
        legend.text = element_text(size=14),
        axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        legend.direction = "horizontal" ,
        legend.position = "top")+scale_fill_manual(
          values = c("darkgoldenrod4", "#365238"))+ labs(fill = "Soil")

boxplot_soil<-boxplot_soil +  geom_text(data = ann_text,label=ann_text$label)

boxplot_soil
```



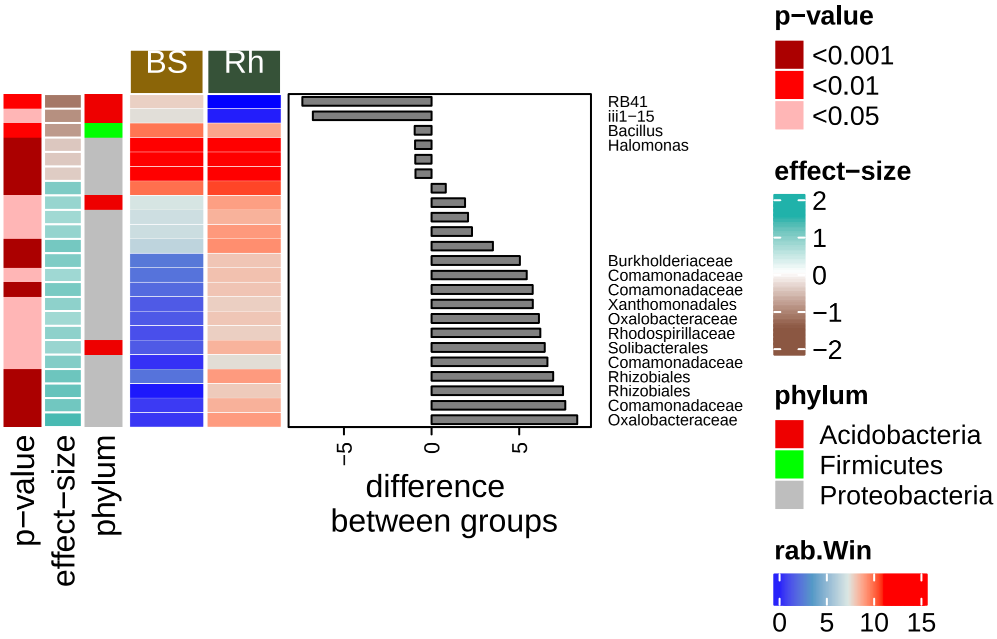
```
#Functional diversity barplot soil
boxplot_soil_f<-func %>%
  ggbarplot(x="fs", y="value", fill = "Soil", add = "mean_se",
            position = position_dodge())+
  theme_bw()+
  labs(y = "Mean functional diversity")+
  facet_wrap(~fs, scales = "free", dir = "v")+
  theme(panel.spacing=unit(1,"lines"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        legend.title = element_text(size = 14),
        legend.text = element_text(size=14),
        axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        legend.direction = "horizontal" ,
        legend.position = "top")+scale_fill_manual(values = c(
          "darkgoldenrod4", "#365238"))+ labs(fill = "Soil")

boxplot_soil_f<-boxplot_soil_f +  geom_text(data = ann_text_f,label=ann_text_f$label)

boxplot_soil_f
```



```
#ggsave('./fig_alpha_soil.png',
 #   width = 2.5, height = 5, dpi = 300, plot = boxplot_soil)

#ggsave('./fig_func_soil.png',
 #      width = 2.5, height = 5, dpi = 300, plot = boxplot_soil_f)

#pdf("fig_alpha_soil.pdf", width=2.5, height=5)
#print(boxplot_soil)
#dev.off()
#pdf("fig_func_soil.pdf", width=2.5, height=5)
#print(boxplot_soil_f)
#dev.off()
```

Aldex results heatmap from Soil

```
#file to heatmap
aldex_all_dif<- read_tsv("../Data/aldex_soil.tsv")

my_fun <- function(x) {
  x %>% separate(
    "Taxon", c("k", "phylum","c", "o","f","g"),
    sep = "\\;", remove = F) %>% dplyr::select(
      Taxon, p.value, effect, diff.btw, rab.win.0, rab.win.1, phylum,
      "FeatureID"="Feature.ID" )%>%
    drop_na(.)%>%
    rownames_to_column(var="rows")%>%
    mutate_all(funs(str_replace(., "k__Bacteria;", "")))%>%
    mutate_all(funs(str_replace(., "; c__; o__; f__; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; o__; f__; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; f__; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; s__", "")))%>%mutate(
      tax= str_extract(Taxon, "[^_]+$")) %>%mutate(
        taxo = paste(rows,"_",tax))%>% mutate_at(
          c(3:7), as.numeric) %>%
    mutate_at(c(3), funs(p.Value = case_when(
      . <= 0.001 ~ "<0.001",
      . >  0.001 & .  <= 0.01 ~ "<0.01",
      . >  0.01 & .  <= 0.05 ~ "<0.05")))%>%
    arrange(diff.btw)%>%column_to_rownames(
      var = "taxo")%>% mutate_at(c(8),funs(str_replace(., "p__", "")))
}
#We are going to multiplicate for -1 in order to change
#the direction of the figure (e.g, bulk soil first and then rhizosphere)

annotation_heatmap <- my_fun(aldex_all_dif) %>% mutate(
  diff.btw2 = diff.btw*-1, effect2 = effect*-1 ) %>% arrange(diff.btw2) %>% mutate(
    taxo= paste(rows,tax, sep = "_"))
data_heatmap<- annotation_heatmap%>%dplyr::select(rab.win.1, rab.win.0) %>% rename(
  rab.win.Rh=rab.win.0 , rab.win.Bs=rab.win.1)


color_heatmap= colorRamp2(seq(min(data_heatmap), max(data_heatmap), length = 5), c(
  "#0000FF","#5499C7", "#DAE7E4",  "red", "#FF0000"))

#Annotation Phylum
cols_ann <- list('phylum' = c(
  " Acidobacteria" = 'red2',
  " Actinobacteria" = 'royalblue',
  " Bacteroidetes"="yellow",
  " Chloroflexi" ="pink",
  " Firmicutes"= "green",
  " Gemmatimonadetes" = "black",
  " Nitrospirae" ="purple",
  " Planctomycetes" ="dark green",
  " Proteobacteria"  ="gray",
  " Verrucomicrobia" ="brown"))
colAnn <- HeatmapAnnotation(phylum = annotation_heatmap$phylum,
                            which = 'row',
                            col = cols_ann,
                            show_legend = T)


#Annotation pvalue

cols_pvalue <- list('p-value' = c("<0.001" = '#AB0000',
                                  "<0.01" = '#FF0000',
                                  "<0.05"="#FFB6B6"))

annP2 = HeatmapAnnotation("p-value" = annotation_heatmap$p.Value,
                          which = "row", col = cols_pvalue,
                          show_legend = T)#, gp = gpar(col = "white"))


#Annotation effect size
effect_col_fun =colorRamp2(c(-1.5, 0, 1.5), c("lightsalmon4", "white", "lightseagreen"))

annEffect = HeatmapAnnotation("effect-size" = annotation_heatmap$effect2,
                              which = "row", col = list("effect-size" = effect_col_fun),
                              show_legend = T,
                              gp = gpar(col = "white"))
# gap = unit(10, "cm"))

#Annotation barplot
bardif= rowAnnotation("difference \n between groups" = anno_barplot(
  annotation_heatmap$diff.btw2, width = unit(4, "cm")))

#Annotation taxonomy


labels = c("RB41", "iii1-15", "Bacillus", "Halomonas", rep("", 7),"Burkholderiaceae",
           "Comamonadaceae","Comamonadaceae", "Xanthomonadales", "Oxalobacteraceae",
           "Rhodospirillaceae", "Solibacterales","Comamonadaceae", "Rhizobiales","Rhizobiales",
           "Comamonadaceae" , "Oxalobacteraceae")

#Heat map
heatmap_aldex_soil<-ComplexHeatmap::  Heatmap(data_heatmap, col = color_heatmap,
row_dend_reorder = F, width = ncol(data_heatmap)*unit(1, "cm"),
height = ncol(data_heatmap)*unit(2.2, "cm"),
left_annotation =  c(annP2, annEffect, colAnn),
cluster_column_slices = F,
heatmap_legend_param = list(direction = "horizontal" ),
right_annotation = c(bardif),
column_split = c("BS", "Rh"),
cluster_rows = F,
cluster_columns = F,
column_km = 1,
column_title_gp = gpar(fill = c("darkgoldenrod4", "#365238" ), col="white"),
border = F, column_gap = unit(0.5, "mm"), row_dend_side = "left",
row_names_side = "right", show_row_names = F,
rect_gp = gpar(col = "white", lwd = 0.2),
row_names_gp = gpar(fontface ="italic", fontsize=10),
show_column_names = F, name = "rab.Win")+
rowAnnotation(labels = anno_text(labels, which = "row",
gpar(col = "black", fontsize = 6)),
width = unit(2, "cm"))

heatmap_aldex_soil
```

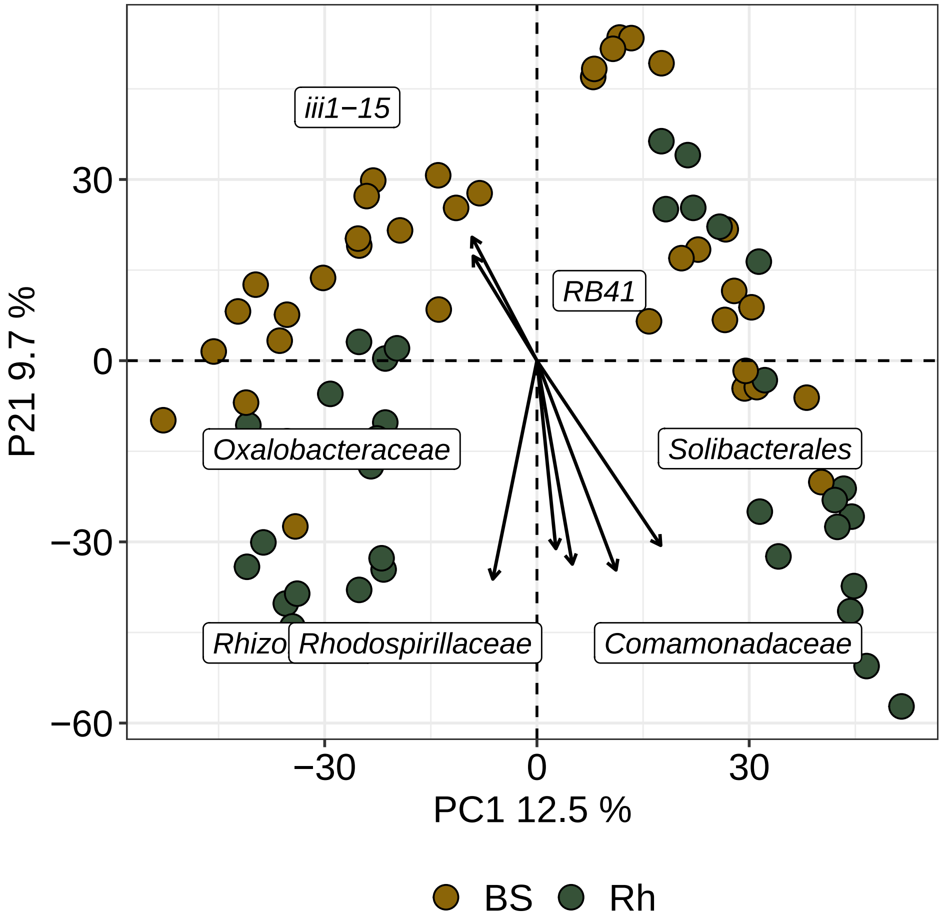


```
#pdf("fig_aldex_soil.pdf", width=6, height=5)
#print(heatmap_aldex_soil)
#dev.off()
```

PCA plot

```
#loading files and formatting

d.pro.0<- read_tsv("../Data/otutable.tsv")%>% column_to_rownames(var = "#OTU ID")
meta<-read_tsv("../Data/metadata.tsv")

meta$Soil<- factor(meta$Soil_sample,
                   levels = c( "bulksoil", "Rhizosphere"),
                   labels = c("BS", "Rh"))

tax<-read_tsv("../Data/taxonomy.tsv") %>% dplyr::select(-Confidence)%>%
  mutate_all(funs(str_replace(., "k__Bacteria;", "")))%>%
  mutate_all(funs(str_replace(., "p__", "")))%>%
  mutate_all(funs(str_replace(., "c__", "")))%>%
  mutate_all(funs(str_replace(., "o__", "")))%>%
  mutate_all(funs(str_replace(., "f__", "")))%>%
  mutate_all(funs(str_replace(., "g__", "")))%>%
  mutate_all(funs(str_replace(., "s__", "")))%>%
  mutate_all(funs(str_replace(., "; ; ;", "")))%>%
  mutate_all(funs(str_replace(., "; ; ", ""))) %>% rename(
    "FeatureID"=`#OTU ID`, Taxon= taxonomy)

tax2<- read_tsv("../Data/taxonomy.tsv") %>% dplyr::select(
  -Confidence) %>% rename(
    "FeatureID"=`#OTU ID`, Taxon= taxonomy)


#transforming data
```

```r
d.pro <- cmultRepl(t(d.pro.0), method="CZM", output="p-counts")
d.clr.abund.codaseq<-codaSeq.clr(x = d.pro,samples.by.row = F)

#run pca
pcx.abund <- prcomp(d.clr.abund.codaseq)

#labels to pca axis

PC1 <- paste("PC1", round(sum(pcx.abund$sdev[1] ^ 2) / mvar(d.clr.abund.codaseq) * 100, 1), "%")
PC2 <- paste("P21", round(sum(pcx.abund$sdev[2] ^ 2) / mvar(d.clr.abund.codaseq) * 100, 1), "%")


#let's choose som of the significant groups from aldex analysis

vars_chosen<- c("14_RB41",
                "3_iii1-15",
                "16_Oxalobacteraceae" ,
                "11_Comamonadaceae",
                "13_Rhizobiales",
                "21_Solibacterales",
                "20_Rhodospirillaceae")
#these ones were chosen from before (some aldex significant groups)

vars_to_choose<- annotation_heatmap %>%  filter(taxo %in% vars_chosen)

vars_choosing<- data.frame(pcx.abund$rotation) %>%  rownames_to_column(var = "FeatureID")%>%
  mutate(a=sqrt(PC1^2+PC2^2)) %>%
  mutate(PC1=PC1*500, PC2=PC2*500) %>% left_join(tax2)%>% dplyr::select(
    Taxon, PC1, PC2, FeatureID)%>%right_join(vars_to_choose, by = "FeatureID")

#create the base plot with only the arrows
pca_soil_arrows<- ggplot() +
  theme_bw() +
  xlab(PC1) +
  ylab(PC2) +
  theme(axis.text = element_text(colour = "black", size = 14),#setting theme
        axis.title = element_text(colour = "black", size = 14),
        legend.text = element_text(size = 14),
        legend.title = element_blank(),
        legend.position = "bottom") +
  geom_point(                              #individuals
    data=data.frame(pcx.abund$x) %>%   rownames_to_column(var = "SampleID")%>%
      left_join(meta, by = "SampleID"),
    aes(x=PC1, y=PC2, fill=Soil),
    shape=21, size=4) +
  geom_vline(xintercept = 0, linetype = 2) +   #lines-cross
  geom_hline(yintercept = 0, linetype = 2) +
  scale_fill_manual(values = c("darkgoldenrod4", "#365238"))+
  ggrepel::geom_label_repel(data = vars_choosing, aes(x=PC1, y=PC2, label= tax),
                            segment.colour = NA, box.padding = 2, fontface="italic")+
  geom_segment(data = vars_choosing,  #arrows and names
               aes(x=0, y=0, xend=PC1, yend=PC2),
               arrow=arrow(length=unit(0.15,"cm")),
                size= 0.6)

pca_soil_arrows
```



```
#pdf("fig_pca_soil.pdf", width=5, height=5)
#print(pca_soil_arrows)
#dev.off()
```

V. TREATMENT FIGURE

Loading libraries

```
library(cowplot)
library(tidyverse)
library(ggpubr)
library(ComplexHeatmap)
library(circlize)
library(viridis)
library(RColorBrewer)
library(grid)
library(CoDaSeq)
library(ggplot2)
require(compositions) # exploratory d ata analysis of compositional data
require(zCompositions) # used for 0 substitution
require(ALDEx2) # used for per-OTU comparisons
library(CoDaSeq)
library(ggrepel)
```

Loadings files and Barplot Text annotations

```
alpha<- read.table("../Data/alpha_diversity") %>% gather(
  q0:q4, key = "q", value = "value") %>% filter(
    q %in% c("q0", "q1", "q2"))%>%mutate(qs= case_when(
  str_detect(q, "q0") ~ "q=0 (species richness)",
  str_detect(q, "q1") ~ "q=1 (frequent species)",
  str_detect(q, "q2") ~ "q=2 (dominant species)"))
head(alpha)
```

```
##   Practice Soil Practice.Location Stage Age Plant Plot ExpUnit  q value
## 1       CA   BS             CA.BS     F   1     1   18 CA.BS.F q0   426
## 2       CA   BS             CA.BS     F   1     2   18 CA.BS.F q0   646
## 3       CA   BS             CA.BS     F   1     3   18 CA.BS.F q0   510
## 4       CA   BS             CA.BS     F   1     1   59 CA.BS.F q0   546
## 5       CA   BS             CA.BS     F   1     2   59 CA.BS.F q0   391
## 6       CA   BS             CA.BS     F   1     3   59 CA.BS.F q0   322
##                         qs
## 1 q=0 (species richness)
## 2 q=0 (species richness)
## 3 q=0 (species richness)
## 4 q=0 (species richness)
## 5 q=0 (species richness)
## 6 q=0 (species richness)
```

```
func<- read.table("../Data/func_MDq.txt") %>% gather(
  MD_q0:MD_q2, key = "q", value = "value")%>%mutate(fs= case_when(
  str_detect(q, "q0") ~ "q=0 (species richness)",
  str_detect(q, "q1") ~ "q=1 (frequent species)",
  str_detect(q, "q2") ~ "q=2 (dominant species)"))
head(func)
```

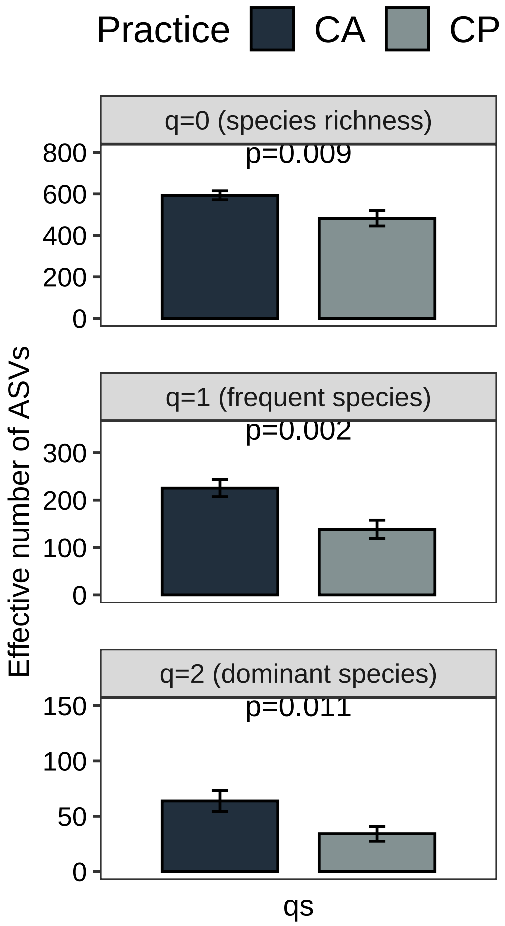
```
##   Practice Soil Practice.Location Stage Age Plant Plot ExpUnit     q    value
## 1       CA   BS             CA.BS     F   1     1   18 CA.BS.F MD_q0 29629.84
## 2       CA   BS             CA.BS     F   1     2   18 CA.BS.F MD_q0 46138.20
## 3       CA   BS             CA.BS     F   1     3   18 CA.BS.F MD_q0 36859.81
## 4       CA   BS             CA.BS     F   1     1   59 CA.BS.F MD_q0 39086.65
## 5       CA   BS             CA.BS     F   1     2   59 CA.BS.F MD_q0 28482.32
## 6       CA   BS             CA.BS     F   1     3   59 CA.BS.F MD_q0 23152.35
##                         fs
## 1 q=0 (species richness)
## 2 q=0 (species richness)
## 3 q=0 (species richness)
## 4 q=0 (species richness)
## 5 q=0 (species richness)
## 6 q=0 (species richness)
```

```
#df with the p values to show in the figures
ann_text<-data.frame(Practice=c("CA", "CA", "CA"),value=c(800,350,150),
qs=c("q=0 (species richness)","q=1 (frequent species)",
"q=2 (dominant species)"),label=c("p=0.009","p=0.002", "p=0.011"))
```

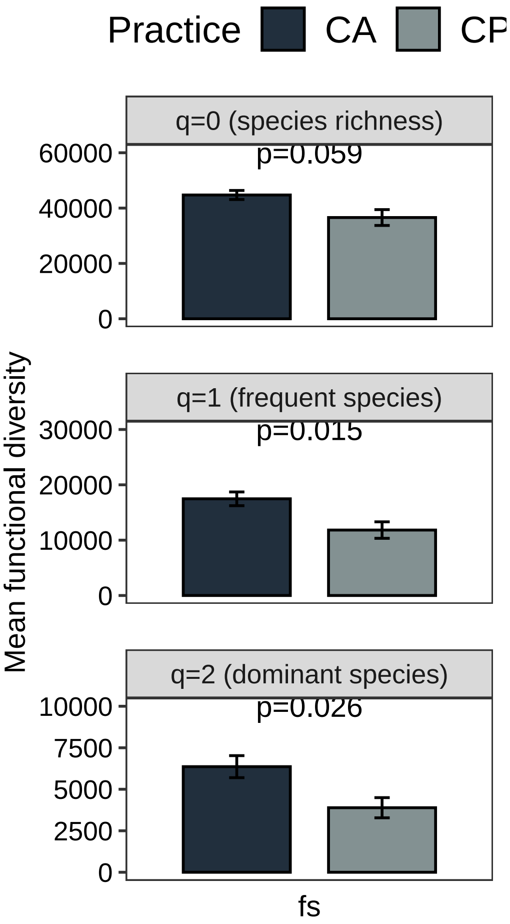
```
#tittles and positiong in y axis

ann_text_f<-data.frame(Practice=c("BS", "BS", "BS"),value=c(60000,30000,10000),
fs=c("q=0 (species richness)","q=1 (frequent species)",
"q=2 (dominant species)"),label=c("p=0.059","p=0.015", "p=0.026"))
#tittles and positiong in y axis
```

Barplots alpha and functional diversity

```
#Alpha diversity barplot soil
boxplot_practice<-alpha %>%
  ggbarplot(x="qs", y="value", fill = "Practice", add = "mean_se",
            position = position_dodge())+
  theme_bw()+
  labs(y = "Effective number of ASVs")+
  facet_wrap(~qs, scales = "free", dir = "v")+
  theme(panel.spacing=unit(1,"lines"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        legend.title = element_text(size = 14),
        legend.text = element_text(size=14),
        axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        legend.direction = "horizontal" ,
        legend.position = "top")+scale_fill_manual(values = c("#212F3D", "#839192"))+ labs(fill = "Prac

boxplot_practice<-boxplot_practice +  geom_text(data = ann_text,label=ann_text$label)

boxplot_practice
```



```
#boxplot
boxplot_practice_f<-func %>%
  ggbarplot(x="fs", y="value", fill = "Practice", add = "mean_se",
            position = position_dodge())+
  theme_bw()+
  labs(y = "Mean functional diversity")+
  facet_wrap(~fs, scales = "free", dir = "v")+
  theme(panel.spacing=unit(1,"lines"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        legend.title = element_text(size = 14),
        legend.text = element_text(size=14),
        axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        legend.direction = "horizontal" ,
        legend.position = "top")+scale_fill_manual(values = c("#212F3D", "#839192"))+ labs(fill = "Prac

boxplot_practice_f<-boxplot_practice_f +  geom_text(data = ann_text_f,label=ann_text_f$label)

boxplot_practice_f
```

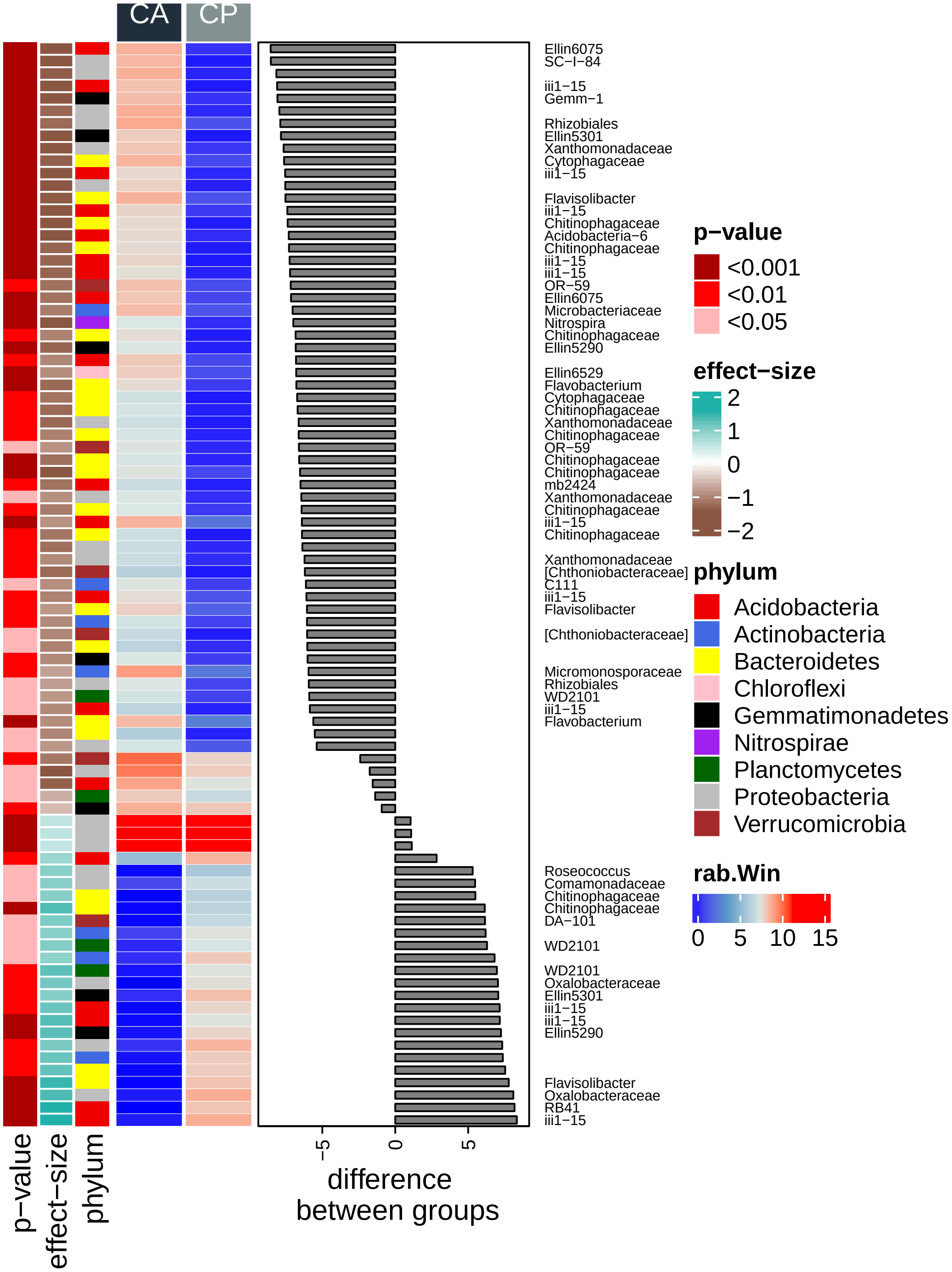


```
#pdf("fig_alpha_practice.pdf", width=2.7, height=5)
#print(boxplot_practice)
#dev.off()
#pdf("fig_func_practice.pdf", width=2.7, height=5)
#print(boxplot_practice_f)
#dev.off()
```

Aldex results heatmap from Soil

```
#file to heatmap
aldex_all_dif<- read_tsv("../Data/aldex_treatment.tsv")

my_fun <- function(x) {
  x %>% separate(
    "Taxon", c("k", "phylum","c", "o","f","g"),
    sep = "\\;", remove = F) %>% dplyr::select(
      Taxon, p.value, effect, diff.btw, rab.win.0, rab.win.1, phylum,
      "FeatureID"="Feature.ID" )%>%
    drop_na(.)%>%
    rownames_to_column(var="rows")%>%
    mutate_all(funs(str_replace(., "k__Bacteria;", "")))%>%
    mutate_all(funs(str_replace(., "; c__; o__; f__; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; o__; f__; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; f__; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; s__", "")))%>%mutate(
      tax= str_extract(Taxon, "[^_]+$")) %>%mutate(
        taxo = paste(rows,"_",tax))%>% mutate_at(
          c(3:7), as.numeric) %>%
    mutate_at(c(3), funs(p.Value = case_when(
      . <= 0.001 ~ "<0.001",
      . >  0.001 & .  <= 0.01 ~ "<0.01",
      . >  0.01 & .  <= 0.05 ~ "<0.05")))%>%
    arrange(diff.btw)%>%column_to_rownames(
      var = "taxo")%>% mutate_at(c(8),funs(str_replace(., "p__", "")))
}
#We are going to multiplicate for -1 in order to change
#the direction of the figure (e.g, bulk soil first and then rhizosphere)

annotation_heatmap <- my_fun(aldex_all_dif) %>%
  rename(rab.win.CA = rab.win.0, rab.win.CP = rab.win.1) %>%
  mutate(taxo= paste(rows,tax, sep = "_"))
data_heatmap<- annotation_heatmap%>%dplyr::select(rab.win.CA, rab.win.CP)

color_heatmap= colorRamp2(seq(min(data_heatmap), max(data_heatmap), length = 5),
                          c("#0000FF","#5499C7", "#DAE7E4",  "red", "#FF0000"))


#Annotation Phylum
cols_ann <- list('phylum' = c(
  " Acidobacteria" = 'red2',
  " Actinobacteria" = 'royalblue',
  " Bacteroidetes"="yellow",
  " Chloroflexi" ="pink",
  " Firmicutes"= "green",
  " Gemmatimonadetes" = "black",
  " Nitrospirae" ="purple",
  " Planctomycetes" ="dark green",
  " Proteobacteria"  ="gray",
  " Verrucomicrobia" ="brown"))
colAnn <- HeatmapAnnotation(phylum = annotation_heatmap$phylum,
                            which = 'row',
                            col = cols_ann,
                            show_legend = T)

cols_pvalue <- list('p-value' = c("<0.001" = '#AB0000',
                                  "<0.01" = '#FF0000',
                                  "<0.05"="#FFB6B6"))

annP2 = HeatmapAnnotation("p-value" = annotation_heatmap$p.Value,
                          which = "row", col = cols_pvalue,
                          show_legend = T)#, gp = gpar(col = "white"))


#Annotation effect size
effect_col_fun =colorRamp2(c(-1.5, 0, 1.5), c("lightsalmon4", "white", "lightseagreen"))
```

```
annEffect = HeatmapAnnotation("effect-size" = annotation_heatmap$effect,
                              which = "row", col = list("effect-size" = effect_col_fun),
                              show_legend = T,
                              gp = gpar(col = "white"))

#Annotation barplot
bardif= rowAnnotation("difference \n between groups" = anno_barplot(
  annotation_heatmap$diff.btw, width = unit(4, "cm")))


#Annotation taxonomy

labels = c("Ellin6075", "SC-I-84", "", "iii1-15" ,  "Gemm-1","",
"Rhizobiales" , "Ellin5301",  "Xanthomonadaceae"  ,
"Cytophagaceae" ,"iii1-15", "" ,"Flavisolibacter" , "iii1-15", "Chitinophagaceae",
"Acidobacteria-6",  "Chitinophagaceae", "iii1-15" , "iii1-15", "OR-59", "Ellin6075",
"Microbacteriaceae","Nitrospira" ,"Chitinophagaceae","Ellin5290",  "","Ellin6529" ,
"Flavobacterium"  , "Cytophagaceae", "Chitinophagaceae","Xanthomonadaceae" ,"Chitinophagaceae",
"OR-59",  "Chitinophagaceae"  ,  "Chitinophagaceae" ,"mb2424",
"Xanthomonadaceae", "Chitinophagaceae"  ,  "iii1-15","Chitinophagaceae" ,""  , "Xanthomonadaceae",
"[Chthoniobacteraceae]",  "C111"  ,  "iii1-15"  , "Flavisolibacter",  "",  "[Chthoniobacteraceae]" ,
"", "" ,"Micromonosporaceae" , "Rhizobiales"  , "WD2101" , "iii1-15" ,
"Flavobacterium", rep("", 11), "Roseococcus"  , "Comamonadaceae" ,  "Chitinophagaceae" ,
"Chitinophagaceae" ,"DA-101" ,  "", "WD2101" , ""  ,"WD2101",
"Oxalobacteraceae", "Ellin5301",  "iii1-15","iii1-15" ,"Ellin5290" , "" ,
"" , "", "Flavisolibacter","Oxalobacteraceae", "RB41","iii1-15")

heatmap_aldex_treatment<-ComplexHeatmap::  Heatmap(data_heatmap, col = color_heatmap, row_dend_reorder =
height = ncol(data_heatmap)*unit(8, "cm"),
left_annotation =  c(annP2, annEffect, colAnn),
cluster_column_slices = F,
 heatmap_legend_param = list(direction = "horizontal" ),
right_annotation = c(bardif),
column_split = rep(c("CA", "CP")),
cluster_rows = F,
cluster_columns = F,
column_km = 1, column_title_gp = gpar(
fill = c("#212F3D", "#839192" ), col="white"),
border = F, column_gap = unit(0.5, "mm"),
row_dend_side = "left",row_names_side = "right",
show_row_names = F ,rect_gp = gpar(col = "white", lwd = 0.2),
row_names_gp = gpar(fontface ="italic", fontsize=10),
show_column_names = F, name = "rab.Win") +
rowAnnotation(labels = anno_text(labels, which = "row", gpar(
  col = "black", fontsize = 6)), width = unit(2, "cm"))

heatmap_aldex_treatment
```

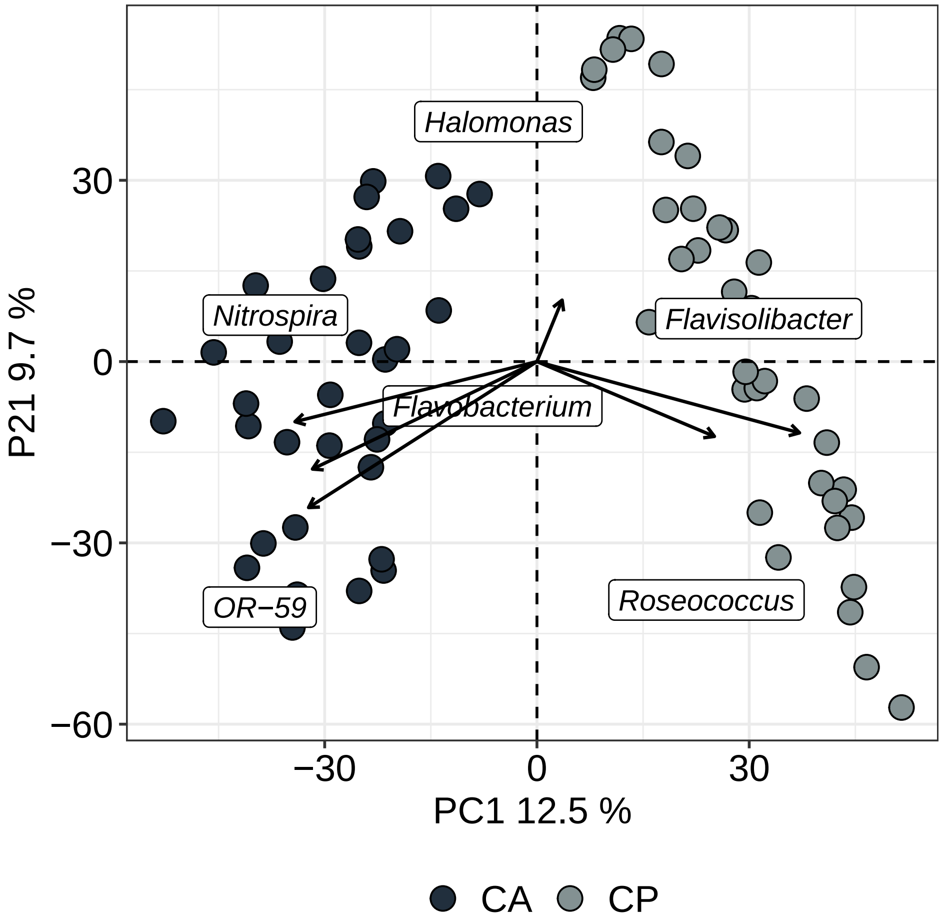


```
#pdf("fig_aldex_TREATMENT.pdf", width=6, height=8)
#print(heatmap_aldex_treatment)
#dev.off()
```

PCA plot

```
#loading files and formatting
```

```r
d.pro.0<- read_tsv("../Data/otutable.tsv")%>% column_to_rownames(var = "#OTU ID")
meta<-read_tsv("../Data/metadata.tsv")

meta$Treatment<- factor(meta$Treatment,
                       levels = c( "AC", "AT"),
                       labels = c("CA", "CP"))

tax<-read_tsv("../Data/taxonomy.tsv") %>% dplyr::select(-Confidence)%>%
  mutate_all(funs(str_replace(., "k__Bacteria;", "")))%>%
  mutate_all(funs(str_replace(., "p__", "")))%>%
  mutate_all(funs(str_replace(., "c__", "")))%>%
  mutate_all(funs(str_replace(., "o__", "")))%>%
  mutate_all(funs(str_replace(., "f__", "")))%>%
  mutate_all(funs(str_replace(., "g__", "")))%>%
  mutate_all(funs(str_replace(., "s__", "")))%>%
  mutate_all(funs(str_replace(., "; ; ;", "")))%>%
  mutate_all(funs(str_replace(., "; ; ", ""))) %>% rename(
    "FeatureID"=`#OTU ID`, Taxon= taxonomy)

tax2<- read_tsv("../Data/taxonomy.tsv") %>% dplyr::select(
  -Confidence) %>% rename(
    "FeatureID"=`#OTU ID`, Taxon= taxonomy)


#transforming data
d.pro <- cmultRepl(t(d.pro.0), method="CZM", output="p-counts")
d.clr.abund.codaseq<-codaSeq.clr(x = d.pro,samples.by.row = F)

#run pca
pcx.abund <- prcomp(d.clr.abund.codaseq)

#labels to pca axis

PC1 <- paste("PC1", round(sum(pcx.abund$sdev[1] ^ 2) / mvar(d.clr.abund.codaseq) * 100, 1), "%")
PC2 <- paste("P21", round(sum(pcx.abund$sdev[2] ^ 2) / mvar(d.clr.abund.codaseq) * 100, 1), "%")


#let's choose som of the significant groups from aldex analysis

vars_chosen<- c("52_Flavisolibacter",
                "37_OR-59",
                "47_Nitrospira" ,
                "16_Halomonas",
                "29_Flavobacterium",
                "   27_Steroidobacter" ,
                "36_Roseococcus")
#these ones were chosen from before (some aldex significant groups)
#these ones were chosen from before (some aldex significant groups)

vars_to_choose<- annotation_heatmap %>%  filter(taxo %in% vars_chosen)

vars_choosing<- data.frame(pcx.abund$rotation) %>%  rownames_to_column(var = "FeatureID")%>%
  mutate(a=sqrt(PC1^2+PC2^2)) %>%
  mutate(PC1=PC1*500, PC2=PC2*500) %>% left_join(tax2)%>% dplyr::select(
    Taxon, PC1, PC2, FeatureID)%>%right_join(vars_to_choose, by = "FeatureID")

#pca plot
pca_treatment_arrows<- ggplot() +
  theme_bw() +
  xlab(PC1) +
  ylab(PC2) +
  theme(axis.text = element_text(colour = "black", size = 14),#setiing theme
        axis.title = element_text(colour = "black", size = 14),
        legend.text = element_text(size = 14),
        legend.title = element_blank(),
        legend.position = "bottom") +
  geom_point(                              #individuals
    data=data.frame(pcx.abund$x) %>%   rownames_to_column(var = "SampleID")%>%
      left_join(meta, by = "SampleID"),
    aes(x=PC1, y=PC2, fill=Treatment),
    shape=21, size=4) +
  geom_vline(xintercept = 0, linetype = 2) +   #lines-cross
  geom_hline(yintercept = 0, linetype = 2) +
  scale_fill_manual(values = c("#212F3D","#839192"))+
  ggrepel::geom_label_repel(data = vars_choosing, aes(x=PC1, y=PC2, label= tax),
                            segment.colour = NA, box.padding = 2, fontface="italic")+
  geom_segment(data = vars_choosing, aes(x=0, y=0, xend=PC1, yend=PC2),
               arrow=arrow(length=unit(0.15,"cm")),
               size= 0.6)

pca_treatment_arrows
```



```
#pdf("fig_pca_treatment.pdf", width=5, height=5)
#print(pca_treatment_arrows)
#dev.off()
```

VI. STAGE FIGURE

Loading libraries

```
library(cowplot)
library(tidyverse)
library(ggpubr)
library(ComplexHeatmap)
library(circlize)
library(viridis)
library(RColorBrewer)
library(grid)
library(CoDaSeq)
library(ggplot2)
require(compositions) # exploratory d ata analysis of compositional data
require(zCompositions) # used for 0 substitution
require(ALDEx2) # used for per-OTU comparisons
library(CoDaSeq)
library(ggrepel)
```

Loadings files and Barplot Text annotations

```
alpha<- read.delim("../Data/Alpha-t_asv_table.txt") %>% gather(
  q0:q4, key = "q", value = "value") %>% filter(
  q %in% c("q0", "q1", "q2"))%>%mutate(qs= case_when(
  str_detect(q, "q0") ~ "q0 (species richness)",
  str_detect(q, "q1") ~ "q1 (frequent species)",
  str_detect(q, "q2") ~ "q2 (dominant species)"))

alpha$Stage <- factor(alpha$Stage,
                      levels = c('V','F', 'G'),ordered = TRUE)

alpha<-alpha%>%arrange(Stage)

head(alpha)
```

```
##            X Practice Soil Practice.Location Stage Age Plant Plot ExpUnit  q
## 1 CAVBS.18.1       CA   BS             CA.BS     V   3     1   18 CA.BS.V q0
## 2 CAVBS.18.2       CA   BS             CA.BS     V   3     2   18 CA.BS.V q0
## 3 CAVBS.18.3       CA   BS             CA.BS     V   3     3   18 CA.BS.V q0
## 4 CAVBS.59.1       CA   BS             CA.BS     V   3     1   59 CA.BS.V q0
## 5 CAVBS.59.2       CA   BS             CA.BS     V   3     2   59 CA.BS.V q0
## 6 CAVBS.59.3       CA   BS             CA.BS     V   3     3   59 CA.BS.V q0
##   value                    qs
## 1   524 q0 (species richness)
## 2   711 q0 (species richness)
## 3   516 q0 (species richness)
## 4   625 q0 (species richness)
## 5   369 q0 (species richness)
## 6   530 q0 (species richness)
```

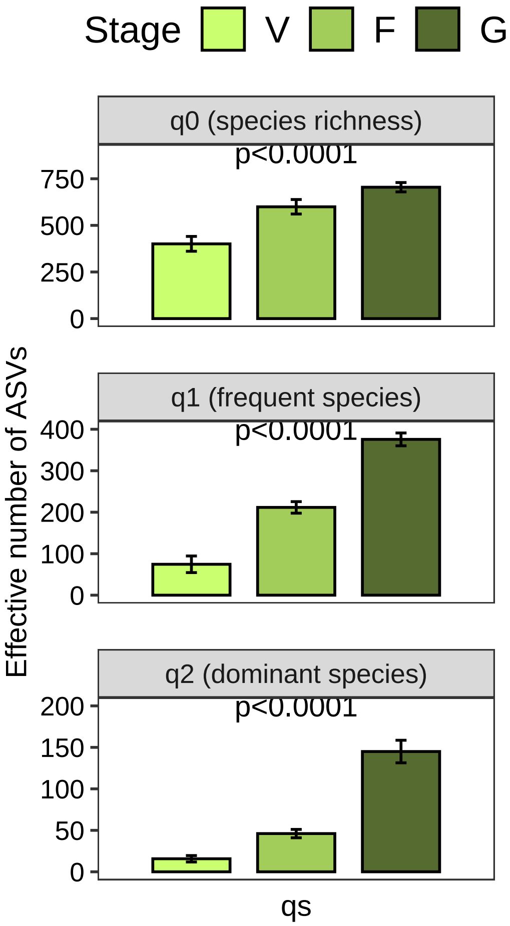
```
func<- read.table("../Data/func_MDq.txt") %>% gather(
  MD_q0:MD_q2, key = "q", value = "value")%>%mutate(fs= case_when(
  str_detect(q, "q0") ~ "q=0 (species richness)",
  str_detect(q, "q1") ~ "q=1 (frequent species)",
  str_detect(q, "q2") ~ "q=2 (dominant species)"))

func$Stage <- factor(func$Stage,
                      levels = c('V','F', 'G'),ordered = TRUE)

func<-func%>%arrange(Stage)

head(func)
```

```
##   Practice Soil Practice.Location Stage Age Plant Plot ExpUnit     q    value
## 1       CA   BS             CA.BS     V   3     1   18 CA.BS.V MD_q0 37655.61
## 2       CA   BS             CA.BS     V   3     2   18 CA.BS.V MD_q0 52669.45
## 3       CA   BS             CA.BS     V   3     3   18 CA.BS.V MD_q0 36080.17
## 4       CA   BS             CA.BS     V   3     1   59 CA.BS.V MD_q0 44486.28
## 5       CA   BS             CA.BS     V   3     2   59 CA.BS.V MD_q0 26415.95
## 6       CA   BS             CA.BS     V   3     3   59 CA.BS.V MD_q0 38715.64
##                       fs
## 1 q=0 (species richness)
## 2 q=0 (species richness)
## 3 q=0 (species richness)
## 4 q=0 (species richness)
## 5 q=0 (species richness)
## 6 q=0 (species richness)
```

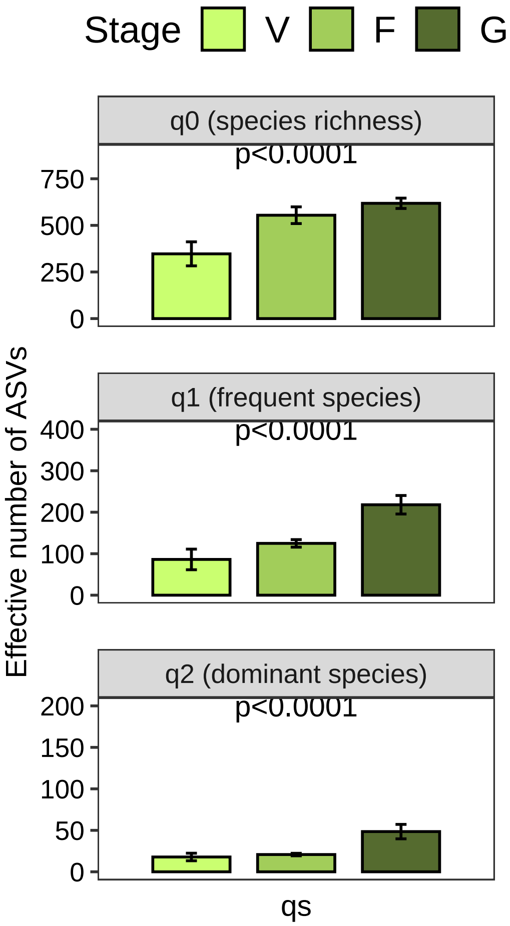
```
#df with the p values to show in the figures
ann_text<-data.frame(Stage=c("G", "G", "G"),value=c(890,400,200),
     qs=c("q0 (species richness)","q1 (frequent species)","q2 (dominant species)"),label=c(
       "p<0.0001","p<0.0001", "p<0.0001")) #tittles and positiong in y axis
#tittles and position in y axis


ann_text_f<-data.frame(Practice=c("G", "G", "G"),value=c(60000,30000,15000),
                       fs=c("q=0 (species richness)","q=1 (frequent species)",
                            "q=2 (dominant species)"),label=c(
                              "p<0.0001","p<0.0001", "p<0.0001"))
#tittles and positiong in y axis
```

Barplots alpha and functional diversity

```
#Alpha diversity barplot
boxplot_rhizo_stage<-subset(alpha, Soil=="Rh") %>%
  ggbarplot(x="qs", y="value", fill = "Stage", add = "mean_se",
            position = position_dodge())+
  theme_bw()+
  labs(y = "Effective number of ASVs")+
  facet_wrap(~qs, scales = "free", dir = "v")+
  theme(panel.spacing=unit(1,"lines"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        legend.title = element_text(size = 14),
        legend.text = element_text(size=14),
        axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        legend.direction = "horizontal" ,
        legend.position = "top")+scale_fill_manual(values = c(
          "darkolivegreen1","darkolivegreen3","darkolivegreen"))+ labs(fill = "Stage")

boxplot_rhizo_stage<-boxplot_rhizo_stage +  geom_text(data = ann_text,label=ann_text$label)

boxplot_rhizo_stage
```

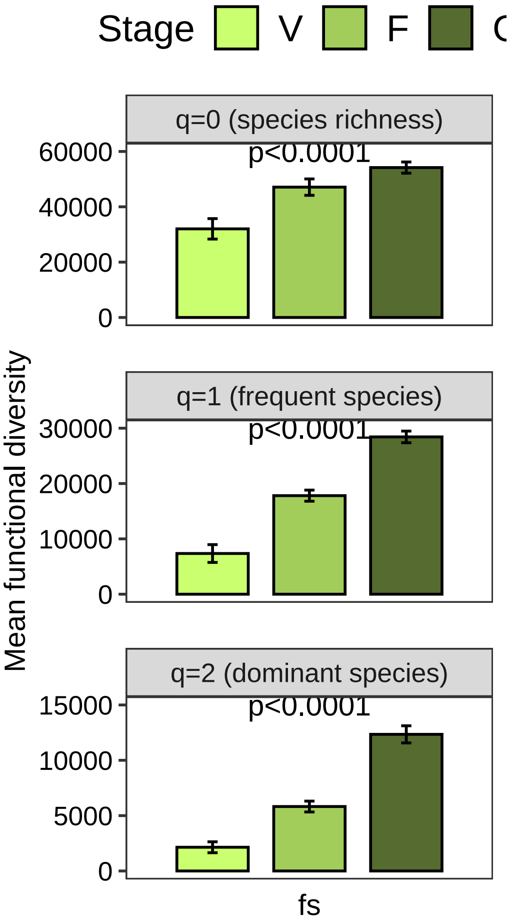


```
boxplot_bulk_stage<-subset(alpha, Soil=="BS") %>%
  ggbarplot(x="qs", y="value", fill = "Stage", add = "mean_se",
            position = position_dodge())+
  theme_bw()+
  labs(y = "Effective number of ASVs")+
  facet_wrap(~qs, scales = "free", dir = "v")+
  theme(panel.spacing=unit(1,"lines"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        legend.title = element_text(size = 14),
        legend.text = element_text(size=14),
        axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        legend.direction = "horizontal" ,
        legend.position = "top")+scale_fill_manual(values = c(
          "darkolivegreen1","darkolivegreen3","darkolivegreen"))+ labs(fill = "Stage")

boxplot_bulk_stage<-boxplot_bulk_stage +  geom_text(data = ann_text,label=ann_text$label)

boxplot_bulk_stage
```

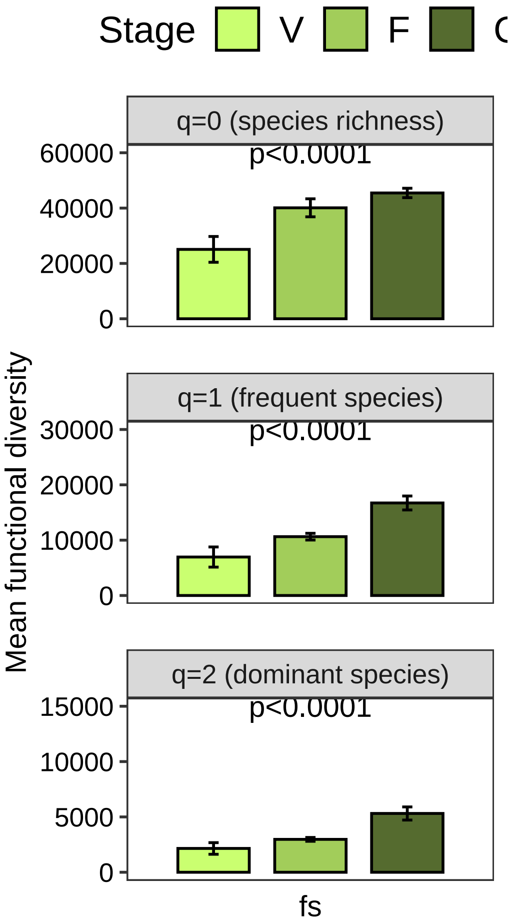


```
#pdf("fig_bulk_stage.pdf", width=2.7, height=5)
#print(boxplot_bulk_stage)
#dev.off()
#pdf("fig_rhizo_stage.pdf", width=2.7, height=5)
#print(boxplot_rhizo_stage)
#dev.off()
```

```
#Functional diversity barplot

boxplot_rhizo_stage_f<-subset(func, Soil=="Rh") %>%
  ggbarplot(x="fs", y="value", fill = "Stage", add = "mean_se",
            position = position_dodge())+
  theme_bw()+
  labs(y = "Mean functional diversity")+
  facet_wrap(~fs, scales = "free", dir = "v")+
  theme(panel.spacing=unit(1,"lines"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        legend.title = element_text(size = 14),
        legend.text = element_text(size=14),
        axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        legend.direction = "horizontal" ,
        legend.position = "top")+scale_fill_manual(values = c("darkolivegreen1","darkolivegreen3","dark
boxplot_rhizo_stage_f<-boxplot_rhizo_stage_f +  geom_text(data = ann_text_f,label=ann_text_f$label)

boxplot_rhizo_stage_f
```



```
boxplot_bulk_stage_f<-subset(func, Soil=="BS") %>%
  ggbarplot(x="fs", y="value", fill = "Stage", add = "mean_se",
            position = position_dodge())+
  theme_bw()+
  labs(y = "Mean functional diversity")+
  facet_wrap(~fs, scales = "free", dir = "v")+
  theme(panel.spacing=unit(1,"lines"),
        strip.text.x = element_text(size = 10),
        axis.text =  element_text(colour = "black", size = 10),
        axis.ticks.x=element_blank(),
        legend.title = element_text(size = 14),
        legend.text = element_text(size=14),
        axis.text.x = element_blank(),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        legend.direction = "horizontal" ,
        legend.position = "top")+scale_fill_manual(values = c("darkolivegreen1","darkolivegreen3","dark

boxplot_bulk_stage_f<-boxplot_bulk_stage_f +  geom_text(data = ann_text_f,label=ann_text_f$label)

boxplot_bulk_stage_f
```

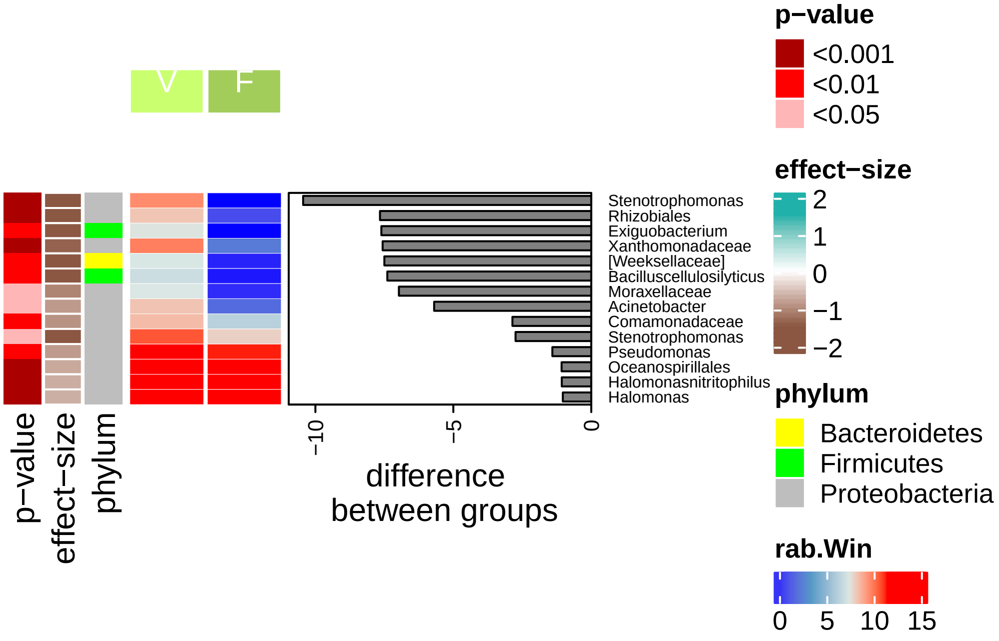


```
#pdf("fig_bulk_stage_f.pdf", width=2.7, height=5)
#print(boxplot_bulk_stage_f)
#dev.off()
#pdf("fig_rhizo_stage_f.pdf", width=2.7, height=5)
#print(boxplot_rhizo_stage_f)
#dev.off()
```

Aldex results heatmap from Soil

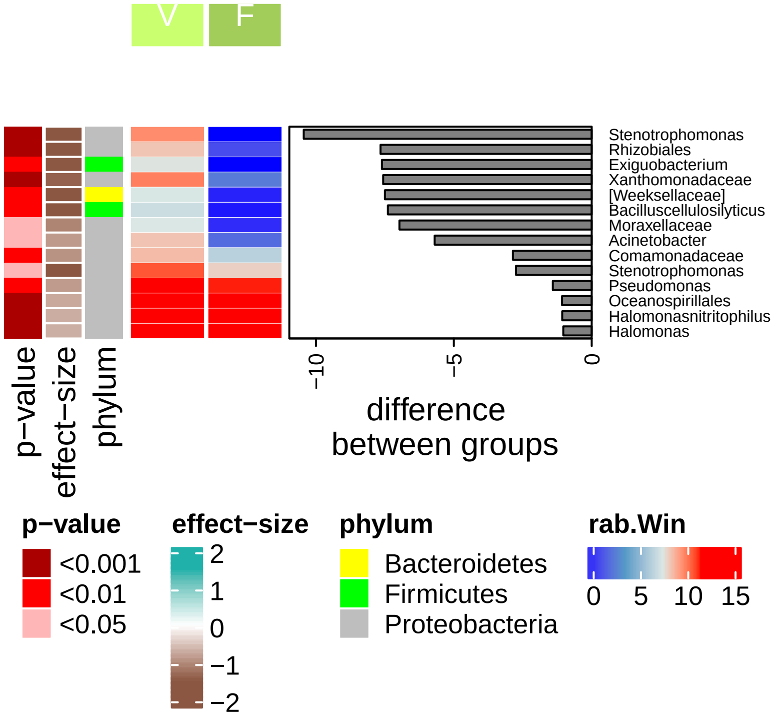
```
#function to heatmap
my_fun <- function(x) {
  x %>% separate(
    "Taxon", c("k", "phylum","c", "o","f","g"),
    sep = "\\;", remove = F) %>% dplyr::select(
      Taxon, p.value, effect, diff.btw, rab.win.0, rab.win.1, phylum,
      "FeatureID"="Feature.ID" )%>%
    drop_na(.)%>%
    rownames_to_column(var="rows")%>%
    mutate_all(funs(str_replace(., "k__Bacteria;", "")))%>%
    mutate_all(funs(str_replace(., "; c__; o__; f__; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; o__; f__; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; f__; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; g__; s__", "")))%>%
    mutate_all(funs(str_replace(., "; s__", "")))%>%mutate(
      tax= str_extract(Taxon, "[^_]+$")) %>%mutate(
        taxo = paste(rows,"_",tax))%>% mutate_at(
          c(3:7), as.numeric) %>%
    mutate_at(c(3), funs(p.Value = case_when(
      . <= 0.001 ~ "<0.001",
      . >  0.001 & .  <= 0.01 ~ "<0.01",
      . >  0.01 & .  <= 0.05 ~ "<0.05")))%>%
    arrange(diff.btw)%>%column_to_rownames(
      var = "taxo")%>% mutate_at(c(8),funs(str_replace(., "p__", "")))}

#VvsF
#file to heatmap
aldex_all_dif_VvsF<- read_tsv("../Data/aldex_all_dif_VvsF.tsv")

annotation_heatmap1 <- my_fun(aldex_all_dif_VvsF)
data_heatmap<- annotation_heatmap1%>%dplyr::select(rab.win.0, rab.win.1)

#Setting colors to heatmap
colo_heatmap= colorRamp2(seq(min(data_heatmap), max(
  data_heatmap), length = 5), c(
    "#0000FF","#5499C7", "#DAE7E4",  "red", "#FF0000"))

#annotation phylum
cols_ann <- list('phylum' = c(" Acidobacteria" = 'red2',
                              " Actinobacteria" = 'royalblue',
                              " Bacteroidetes"="yellow",
                              " Chloroflexi" ="pink",
                              " Firmicutes"= "green",
                              " Gemmatimonadetes" = "black",
                              " Proteobacteria"  ="gray",
                              " Verrucomicrobia" ="brown",
                              " Nitrospirae" ="DarkGreen",
                              " TM7"= "blue",
                              " Planctomycetes" ="purple"))
colAnn <- HeatmapAnnotation(phylum = annotation_heatmap1$phylum,
                            which = 'row',
                            col = cols_ann,
                            show_legend = T)

#pvalue annotation

cols_pvalue <- list('p-value' = c("<0.001" = '#AB0000',
                                  "<0.01" = '#FF0000',
                                  "<0.05"="#FFB6B6"))

annP2 = HeatmapAnnotation("p-value" = annotation_heatmap1$p.Value,
                          which = "row", col = cols_pvalue,
                          show_legend = T)#, gp = gpar(col = "white"))


#effect annotation
effect_col_fun =colorRamp2(c(-1.5, 0, 1.5), c(
  "lightsalmon4", "white", "lightseagreen"))
```

```
annEffect = HeatmapAnnotation("effect-size" = annotation_heatmap1$effect,
                              which = "row", col = list("effect-size" = effect_col_fun),
                              show_legend = T,
                              gp = gpar(col = "white"))
#barplot annotation
bardif= rowAnnotation(
  "difference \n between groups" = anno_barplot(
    annotation_heatmap1$diff.btw, width = unit(4, "cm")))

labels1 = (annotation_heatmap1$tax)

htVvsF<-  ComplexHeatmap::Heatmap(
  as.matrix(data_heatmap), col = colo_heatmap, row_dend_reorder = F,
  height = ncol(data_heatmap)*unit(1.4, "cm"),
  left_annotation =  c(annP2,annEffect, colAnn),
  heatmap_legend_param = list(direction = "horizontal" ),
  right_annotation = c(bardif),
  column_split = factor(rep(c("V", "F")), levels = c("V", "F")),
  cluster_rows = F, column_km = 1,
  column_title_gp = gpar(fill = c("darkolivegreen1","darkolivegreen3"), col="white"),
  border = F, column_gap = unit(0.5, "mm"), row_dend_side = "left",
  row_names_side = "right",show_row_names = F ,
  rect_gp = gpar(col = "white", lwd = 0.2), row_names_gp = gpar(
  fontface ="italic", fontsize=10),show_column_names = F, name = "rab.Win",
  cluster_column_slices = F) +rowAnnotation(labels = anno_text(
  labels1, which = "row", gpar(col = "black", fontsize = 6)),width = unit(2, "cm"))


htVvsF
```

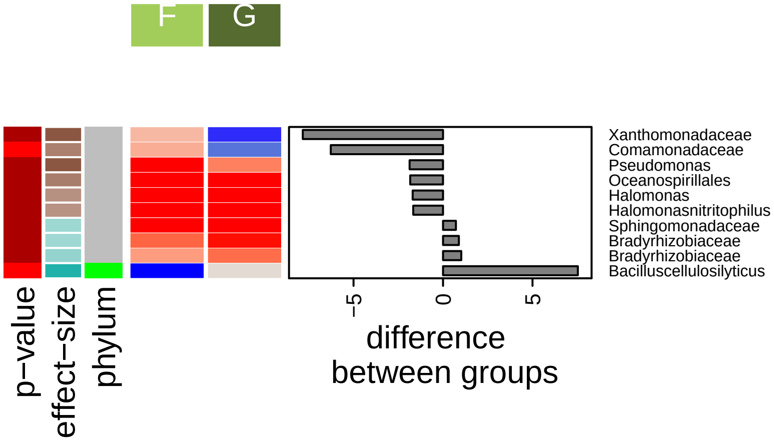


```
#pdf("fig_aldex_VvsF.pdf", width=6, height=5)
#print(htVvsF)
#dev.off()
```

```
htVvsF.2<-draw(htVvsF, heatmap_legend_side = "bottom",
                annotation_legend_side = "bottom")
```



```
#pdf("fig_aldex_VvsF2.pdf", width=6, height=6)
#print(htVvsF.2)
#dev.off()


#FVSG
#loading file
aldex_all_dif_FvsG<-read_tsv("../Data/aldex_all_dif_FvsG.tsv")

annotation_heatmap2 <- my_fun(aldex_all_dif_FvsG)
data_heatmap<- annotation_heatmap2%>%dplyr::select(rab.win.0, rab.win.1)

#Setting colors to heatmap
colo_heatmap= colorRamp2(seq(min(data_heatmap), max(data_heatmap),
length = 5), c("#0000FF","#5499C7", "#DAE7E4",  "red", "#FF0000"))

#annotation phylum
cols_ann <- list('phylum' = c(
  " Acidobacteria" = 'red2',
  " Actinobacteria" = 'royalblue',
  " Bacteroidetes"="yellow",
  " Chloroflexi" ="pink",
  " Firmicutes"= "green",
  " Gemmatimonadetes" = "black",
  " Proteobacteria"  ="gray",
  " Verrucomicrobia" ="brown",
  " TM7"= "blue",
  " Planctomycetes" ="purple"))

colAnn <- HeatmapAnnotation(phylum = annotation_heatmap2$phylum,
                            which = 'row',
                            col = cols_ann,
                            show_legend = F)


#pvalue annotation

cols_pvalue <- list('p-value' = c("<0.001" = '#AB0000',
                                  "<0.01" = '#FF0000',
                                  "<0.05"="#FFB6B6"))

annP2 = HeatmapAnnotation("p-value" = annotation_heatmap2$p.Value,
                          which = "row", col = cols_pvalue,
                          show_legend = F)

#effect annotation
effect_col_fun =colorRamp2(c(-1.5, 0, 1.5), c(
  "lightsalmon4", "white", "lightseagreen"))

annEffect = HeatmapAnnotation("effect-size" = annotation_heatmap2$effect,
                              which = "row", col = list(
                                "effect-size" = effect_col_fun),
                              show_legend = F,
                              gp = gpar(col = "white"))

#barplot annotation
bardif= rowAnnotation(
  "difference \n between groups" = anno_barplot(
    annotation_heatmap2$diff.btw, width = unit(4, "cm")))

labels2 = (annotation_heatmap2$tax)

htFvsG<-ComplexHeatmap::Heatmap(
  data_heatmap, col = colo_heatmap, row_dend_reorder = F,
  width = ncol(data_heatmap)*unit(1, "cm"),
  height = ncol(data_heatmap)*unit(1, "cm"),
  left_annotation =  c(annP2, annEffect, colAnn),
  heatmap_legend_param = list(direction = "horizontal" ),
  right_annotation = c(bardif),
  column_split = rep(c("F", "G")),
  cluster_rows = F, show_heatmap_legend = F,
  cluster_column_slices = F,
  column_km = 1, column_title_gp = gpar(
  fill = c("darkolivegreen3","darkolivegreen"), col="white"),
  border = F, column_gap = unit(0.5, "mm"),
  row_dend_side = "left",row_names_side = "right",show_row_names = F ,
  rect_gp = gpar(col = "white", lwd = 0.2), row_names_gp = gpar(
  fontface ="italic", fontsize=10),show_column_names = F,
  name = "rab.Win")+  rowAnnotation(
    labels = anno_text(labels2, which = "row", gpar(
  col = "black", fontsize = 6)), width = unit(2, "cm"))

htFvsG
```



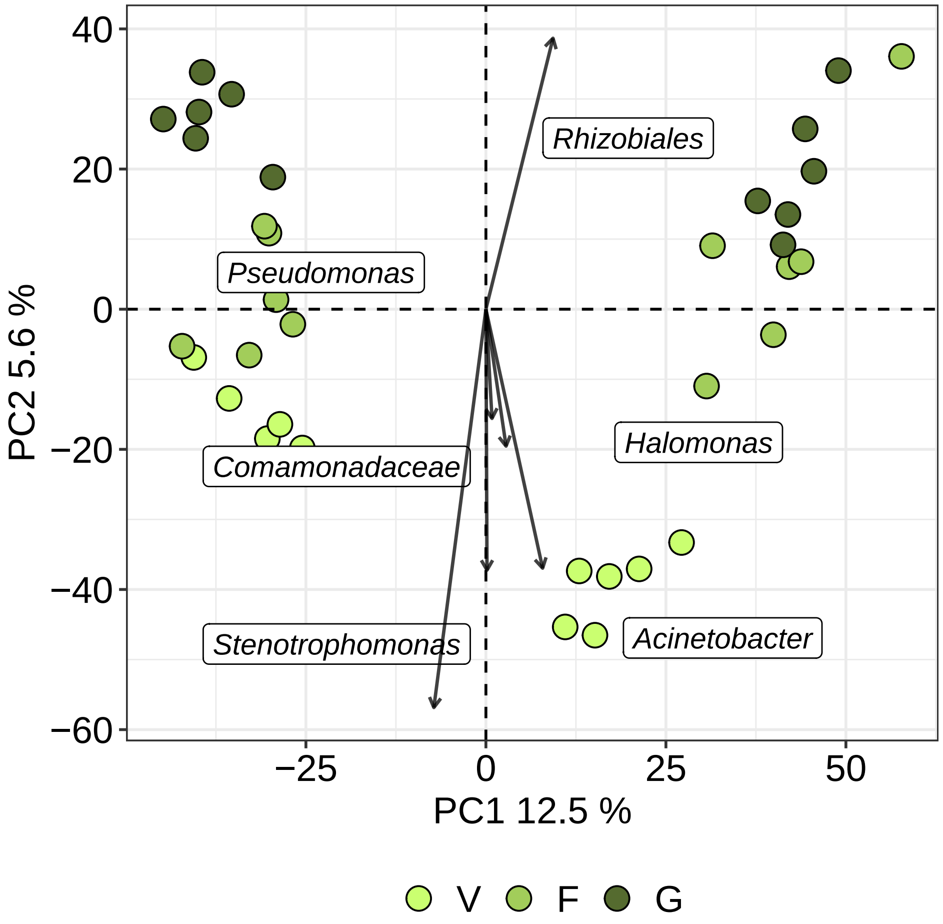
```
#pdf("fig_aldex_FvsG.pdf", width=6, height=5)
#print(htFvsG)
#dev.off()

# VvsG

aldex_all_dif_VvsG<-read_tsv("../Data/aldex_all_dif_VvsG.tsv")
annotation_heatmap3 <- my_fun(aldex_all_dif_VvsG)
data_heatmap<- annotation_heatmap3%>%dplyr::select(rab.win.0, rab.win.1)

#Setting colors to heatmap
colo_heatmap= colorRamp2(seq(min(data_heatmap), max(data_heatmap),
length = 5), c("#0000FF","#5499C7", "#DAE7E4",  "red", "#FF0000"))

#annotation phylum
cols_ann <- list('phylum' = c(
  " Acidobacteria" = 'red2',
  " Actinobacteria" = 'royalblue',
  " Bacteroidetes"="yellow",
  " Chloroflexi" ="pink",
  " Firmicutes"= "green",
  " Gemmatimonadetes" = "black",
  " Proteobacteria"  ="gray",
  " Verrucomicrobia" ="brown",
  " TM7"= "blue",
  " Planctomycetes" ="purple"))

colAnn <- HeatmapAnnotation(phylum = annotation_heatmap3$phylum,
                            which = 'row',
                            col = cols_ann,
                            show_legend = F)

#pvalue annotation

cols_pvalue <- list('p-value' = c("<0.001" = '#AB0000',
                                  "<0.01" = '#FF0000',
                                  "<0.05"="#FFB6B6"))

annP2 = HeatmapAnnotation("p-value" = annotation_heatmap3$p.Value,
                          which = "row", col = cols_pvalue,
                          show_legend = F)#, gp = gpar(col = "white"))


#effect annotation
effect_col_fun =colorRamp2(c(-1.5, 0, 1.5), c(
  "lightsalmon4", "white", "lightseagreen"))

annEffect = HeatmapAnnotation("effect-size" = annotation_heatmap3$effect,
                              which = "row",
                              col = list("effect-size" = effect_col_fun),
                              show_legend = F,
                              gp = gpar(col = "white"))
#barplot annotation
bardif= rowAnnotation(
  "difference \n between groups" = anno_barplot(
    annotation_heatmap3$diff.btw, width = unit(4, "cm")))

labels3 = (annotation_heatmap3$tax)

htVvsG<-ComplexHeatmap::Heatmap(
  data_heatmap, col = colo_heatmap, row_dend_reorder = F,
  width = ncol(data_heatmap)*unit(1, "cm"),
  height = ncol(data_heatmap)*unit(1.4, "cm"),
  left_annotation =  c(annP2, annEffect, colAnn),
  heatmap_legend_param = list(direction = "horizontal" ),
  right_annotation = c(bardif),
  column_split = factor(rep(c("V", "G")), levels = c("V", "G")),
  cluster_rows = F,show_heatmap_legend = F,
  column_km = 1, column_title_gp = gpar(fill = c(
 "darkolivegreen1","darkolivegreen"), col="white"),
 border = F, column_gap = unit(0.5, "mm"),
 row_dend_side = "left",row_names_side = "right",show_row_names = F ,
 rect_gp = gpar(col = "white", lwd = 0.2), row_names_gp = gpar(
fontface ="italic", fontsize=10),show_column_names = F, name = "rab.Win")+
rowAnnotation(labels = anno_text(labels3, which = "row",
gpar(col = "black", fontsize = 6)),width = unit(2, "cm"))

#pdf("fig_aldex_VvsG.pdf", width=6, height=5)
#print(htVvsG)
#dev.off()
```

PCA plot

```
#loading files and formatting
d.pro.0<- read_tsv("../Data/otutable.tsv") %>% column_to_rownames(var = "#OTU ID")

meta<-read_tsv("../Data/metadata.tsv")
```

```
meta$Stage<- factor(meta$Maize_development_stage,
                    levels = c( "Vegetative", "Flowering", "Grainfilling"),
                    labels = c("V", "F", "G"))

tax2<- read_tsv("../Data/taxonomy.tsv")%>% rename(
    "FeatureID"=`#OTU ID`, Taxon= taxonomy)

tax3<-tax2%>% separate(
  "Taxon", c("k", "phylum","c", "o","f","g"),
  sep = "\\;", remove = F) %>%
  rownames_to_column(var="rows")%>%
  mutate_all(funs(str_replace(., "k__Bacteria;", "")))%>%
  mutate_all(funs(str_replace(., "; c__; o__; f__; g__; s__", "")))%>%
  mutate_all(funs(str_replace(., "; o__; f__; g__; s__", "")))%>%
  mutate_all(funs(str_replace(., "; f__; g__; s__", "")))%>%
  mutate_all(funs(str_replace(., "; g__; s__", "")))%>%
  mutate_all(funs(str_replace(., "; s__", "")))%>%mutate(
    tax= str_extract(Taxon, "[^_]+$"))

sample_to_choose<- meta %>% filter(Soil_sample=="Rhizosphere")

#transforming data
d.pro.0.rhizo<- d.pro.0  %>% dplyr::select(0, sample_to_choose$SampleID)
d.pro.rhizo <- t(cmultRepl(t(d.pro.0.rhizo), method="CZM", output="p-counts"))
d.clr.abund.codaseq.rhizo<-codaSeq.clr(x = d.pro.rhizo,samples.by.row = F)

#run pca
pcx.abund.rhizo <- prcomp(t(d.clr.abund.codaseq.rhizo))

#labels to pca axis

PC1 <- paste(
  "PC1", round(sum(pcx.abund.rhizo$sdev[1] ^ 2) / mvar(d.clr.abund.codaseq.rhizo) , 1), "%")
PC2 <- paste(
  "PC2", round(sum(pcx.abund.rhizo$sdev[2] ^ 2) / mvar(d.clr.abund.codaseq.rhizo) , 1), "%")

#let's choose som of the significant groups from aldex analysis

annot_heat<- merge(annotation_heatmap1,
                   annotation_heatmap2, by = "FeatureID") %>%full_join(
                     annotation_heatmap3, by = "FeatureID")

vars_chosen<- c("d0dbf2a66c655edf1f45eb0fe9415866",
                "2553e8df6ec901e443d9f4ed5f7ea2fe",
                "008e9d51155f32838e58a5a6eb48f335" ,
                #"61d320df173b3b20ac4bb8a0b9adcb3c",
                "f35cd29ecc2c92909b596ad30084ea48",
                "f75c3dab2258512ada2c3af6f86e5865",
                "cf75802eef23e2082bcb012af233a01b")
                # "3882df43374c4d647c02bb95fc46c3ed",
                #"2553e8df6ec901e443d9f4ed5f7ea2fe",
                #"087cf9bebbcc26a354bc475125443455")
#these ones were chosen from before (some aldex significant groups)
```

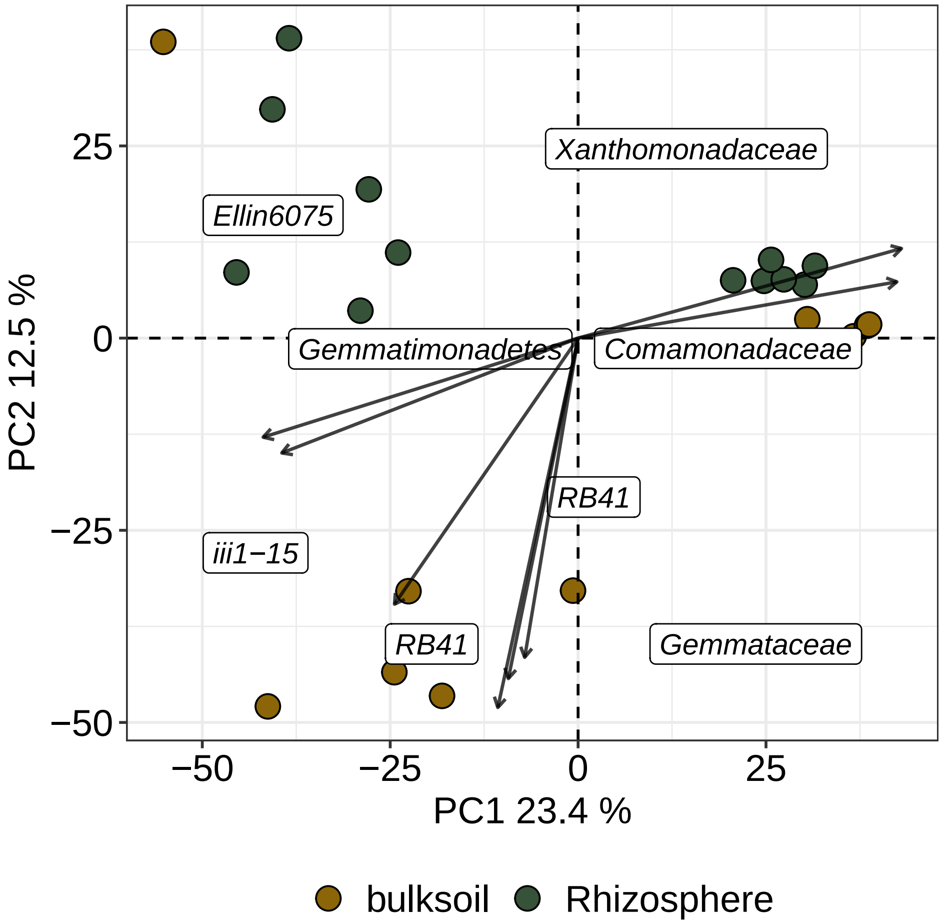
```
vars_to_choose<- annotation_heatmap3 %>%  rownames_to_column(
  var = "ids")%>%filter(FeatureID %in% vars_chosen)

vars_choosing<- data.frame(
  pcx.abund.rhizo$rotation) %>%  rownames_to_column(
    var = "FeatureID")%>%
  mutate(a=sqrt(PC1^2+PC2^2)) %>%
  mutate(PC1=PC1*500, PC2=PC2*500) %>% dplyr::select(
    PC1, PC2, FeatureID)%>%right_join(vars_to_choose, by = "FeatureID")

#pca-plot
pca_stage_arrows<- ggplot() +
  theme_bw() +
  xlab(PC1) +
  ylab(PC2) +
    theme(axis.text = element_text(colour = "black", size = 14), #setting themes
        axis.title = element_text(colour = "black", size = 14),
        legend.text = element_text(size = 14),
        legend.title = element_blank(),
        legend.position = "bottom",
        legend.box = "horizontal",
        legend.direction = "horizontal") +
  geom_point(                              #individuals
    data=data.frame(pcx.abund.rhizo$x) %>%   rownames_to_column(var = "SampleID")%>%
    left_join(meta, by = "SampleID"),
    aes(x=PC1, y=PC2, fill=Stage),
    shape=21, size=4) +
  geom_vline(xintercept = 0, linetype = 2) +   #lines-cross
  geom_hline(yintercept = 0, linetype = 2) +
  scale_fill_manual(values = c( "darkolivegreen1","darkolivegreen3","darkolivegreen"))+
  ggrepel::geom_label_repel(data = vars_choosing, aes(x=PC1, y=PC2, label= tax),
                            segment.colour = NA, box.padding = 2, fontface="italic")+
  geom_segment(data = vars_choosing, aes(x = 0, y = 0, xend = PC1, yend = PC2),
               arrow=arrow(length=unit(0.15,"cm")), #arros and names
               alpha = 0.75, color = 'black', size= 0.6)

pca_stage_arrows
```



```
#pdf("fig_PCA_rhizo_stage.pdf", width=5, height=5)
#print(pca_stage_arrows)
#dev.off()
```

```
# PCA VEGETATIVE STAGE

sample_to_choose_v<- meta %>% filter(Stage=="V")
d.pro.0.V<- d.pro.0  %>% dplyr::select(0, sample_to_choose_v$SampleID)
d.pro.V <- t(cmultRepl(t(d.pro.0.V), method="CZM", output="p-counts")) #tratamiento de 0

d.clr.abund.codaseq.V<-codaSeq.clr(x = d.pro.V, samples.by.row = F) #transformacion clr

pcx.abund.V <- prcomp(t(d.clr.abund.codaseq.V))



PC1 <- paste(
  "PC1", round(sum(pcx.abund.V$sdev[1] ^ 2) / mvar(d.clr.abund.codaseq.V), 1), "%")
PC2 <- paste(
  "PC2", round(sum(pcx.abund.V$sdev[2] ^ 2) / mvar(d.clr.abund.codaseq.V) , 1), "%")

vars_choosing<- data.frame(pcx.abund.V$rotation) %>%  rownames_to_column(var = "FeatureID")%>%
  mutate(a=sqrt(PC1^2+PC2^2)) %>%
  mutate(PC1=PC1*500, PC2=PC2*500) %>% top_n(8, a) %>% dplyr::select(
    PC1, PC2, FeatureID) %>% right_join(tax3, by = "FeatureID")
```

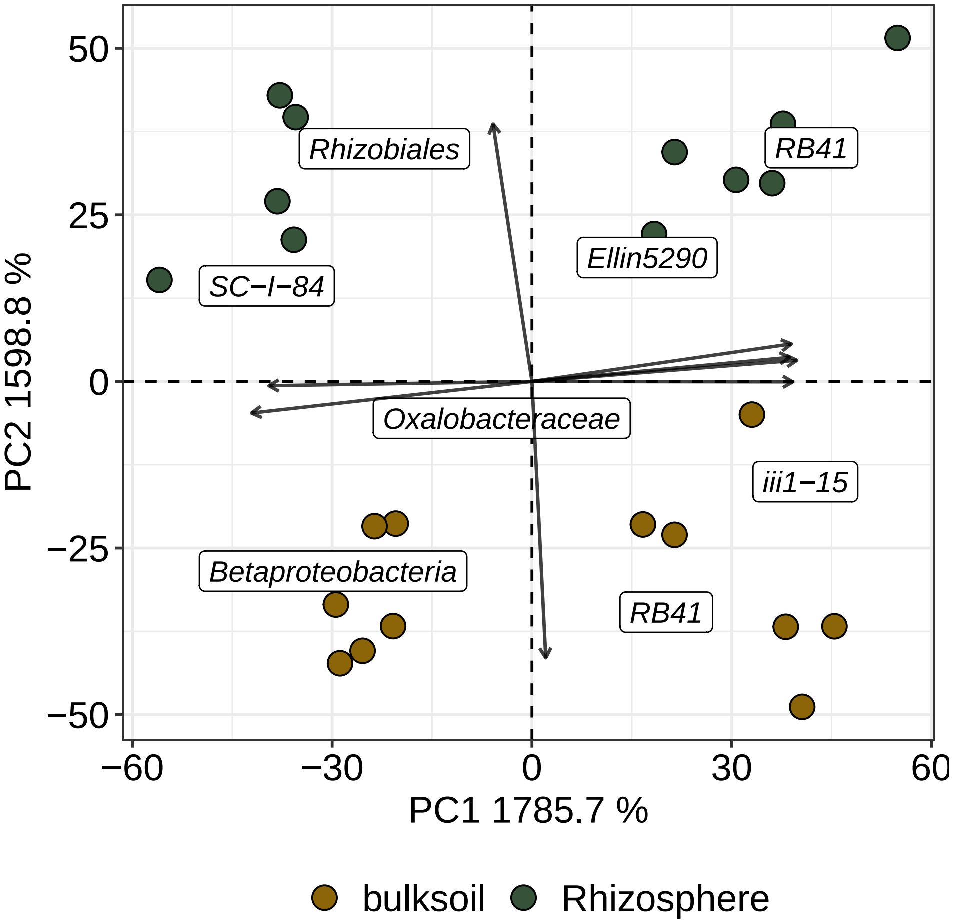
```r
#pca-plot
pca_stage_arrows_V<- ggplot() +
  theme_bw() +
  xlab(PC1) +
  ylab(PC2) +
  theme(axis.text = element_text(colour = "black", size = 14), #setting theme
        axis.title = element_text(colour = "black", size = 14),
        legend.text = element_text(size = 14),
        legend.title = element_blank(),
        legend.position = "bottom",
        legend.box = "horizontal",
        legend.direction = "horizontal") +
  geom_point(                              #individuals
    data=data.frame(pcx.abund.V$x) %>%   rownames_to_column(var = "SampleID")%>%
      left_join(meta, by = "SampleID"),
    aes(x=PC1, y=PC2, fill=Soil_sample),
    shape=21, size=4) +
  geom_vline(xintercept = 0, linetype = 2) +   #lines-cross
  geom_hline(yintercept = 0, linetype = 2) +
  scale_fill_manual(values = c("darkgoldenrod4", "#365238"))+
  ggrepel::geom_label_repel(data = vars_choosing, aes(x=PC1, y=PC2, label= tax),
                            segment.colour = NA, box.padding = 2, fontface="italic")+
  geom_segment(data = vars_choosing, aes(x = 0, y = 0, xend = PC1, yend = PC2),
               arrow=arrow(length=unit(0.15,"cm")), #arrows and names
               alpha = 0.75, color = 'black', size= 0.6)

pca_stage_arrows_V
```



```
#pdf("fig_PCA_vegetative.pdf", width=5, height=5)
#print(pca_stage_arrows_V)
#dev.off()
```

```
# PCA FLOWERING STAGE

sample_to_choose_f<- meta %>% filter(Stage=="F")
d.pro.0.F<- d.pro.0  %>% dplyr::select(0, sample_to_choose_f$SampleID)
d.pro.F <- t(cmultRepl(t(d.pro.0.F), method="CZM", output="p-counts")) #tratamiento de 0

d.clr.abund.codaseq.F<-codaSeq.clr(x = d.pro.F, samples.by.row = F) #transformacion clr

pcx.abund.F <- prcomp(t(d.clr.abund.codaseq.F))


PC1 <- paste(
  "PC1", round(sum(pcx.abund.F$sdev[1] ^ 2) / mvar(d.clr.abund.codaseq.F) * 100, 1), "%")
PC2 <- paste(
  "PC2", round(sum(pcx.abund.F$sdev[2] ^ 2) / mvar(d.clr.abund.codaseq.F) * 100, 1), "%")

vars_choosing<- data.frame(pcx.abund.F$rotation) %>%  rownames_to_column(var = "FeatureID")%>%
  mutate(a=sqrt(PC1^2+PC2^2)) %>%
  mutate(PC1=PC1*500, PC2=PC2*500) %>% top_n(8, a) %>% dplyr::select(
    PC1, PC2, FeatureID) %>% right_join(tax3, by = "FeatureID")
```

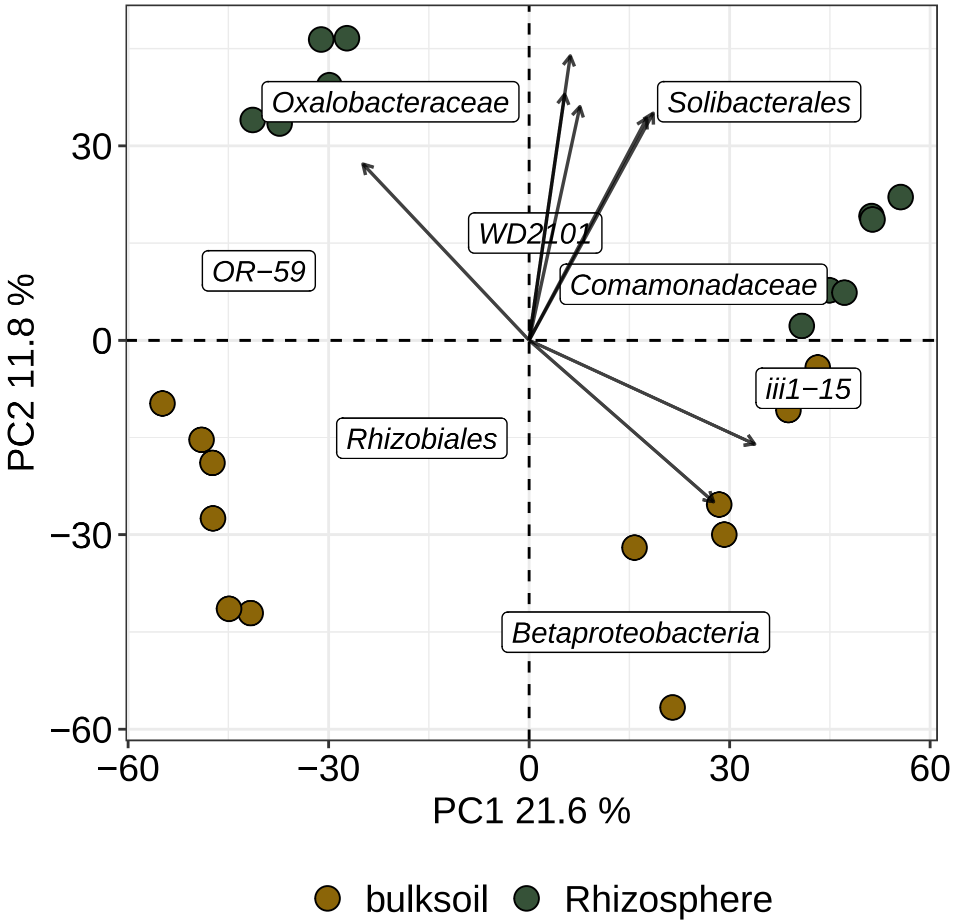
```
#create the base plot with only the arrows
pca_stage_arrows_F<- ggplot() +
  theme_bw() +
  xlab(PC1) +
  ylab(PC2) +
  theme(axis.text = element_text(colour = "black", size = 14), #setting themes
        axis.title = element_text(colour = "black", size = 14),
        legend.text = element_text(size = 14),
        legend.title = element_blank(),
        legend.position = "bottom",
        legend.box = "horizontal",
        legend.direction = "horizontal") +
  geom_point(                              #individuals
    data=data.frame(pcx.abund.F$x) %>%   rownames_to_column(var = "SampleID")%>%
      left_join(meta, by = "SampleID"),
    aes(x=PC1, y=PC2, fill=Soil_sample),
    shape=21, size=4) +
  geom_vline(xintercept = 0, linetype = 2) +   #lines-cross
  geom_hline(yintercept = 0, linetype = 2) +
  scale_fill_manual(values = c("darkgoldenrod4", "#365238"))+
  ggrepel::geom_label_repel(data = vars_choosing, aes(x=PC1, y=PC2, label= tax),
                            segment.colour = NA, box.padding = 2, fontface="italic")+
  geom_segment(data = vars_choosing, aes(x = 0, y = 0, xend = PC1, yend = PC2),
               arrow=arrow(length=unit(0.15,"cm")), #arrows and names
               alpha = 0.75, color = 'black', size= 0.6)

pca_stage_arrows_F
```



```
#pdf("fig_PCA_flowering.pdf", width=5, height=5)
#print(pca_stage_arrows_F)
#dev.off()
```

```
# PCA GRAIN FILLING STAGE

sample_to_choose_g<- meta %>% filter(Stage=="G")
d.pro.0.G<- d.pro.0  %>% dplyr::select(0, sample_to_choose_g$SampleID)
d.pro.G <- t(cmultRepl(t(d.pro.0.G), method="CZM", output="p-counts")) #tratamiento de 0

d.clr.abund.codaseq.G<-codaSeq.clr(x = d.pro.G, samples.by.row = F) #transformacion clr

pcx.abund.G <- prcomp(t(d.clr.abund.codaseq.G))



PC1 <- paste("PC1", round(sum(pcx.abund.G$sdev[1] ^ 2) / mvar(d.clr.abund.codaseq.G) , 1), "%")
PC2 <- paste("PC2", round(sum(pcx.abund.G$sdev[2] ^ 2) / mvar(d.clr.abund.codaseq.G) , 1), "%")

vars_choosing<- data.frame(pcx.abund.G$rotation) %>%  rownames_to_column(var = "FeatureID")%>%
  mutate(a=sqrt(PC1^2+PC2^2)) %>%
  mutate(PC1=PC1*500, PC2=PC2*500) %>% top_n(8, a) %>% dplyr::select(
    PC1, PC2, FeatureID) %>% right_join(tax3, by = "FeatureID")

#create the base plot with only the arrows
```

```
pca_stage_arrows_G<- ggplot() +
  theme_bw() +
  xlab(PC1) +
  ylab(PC2) +
  theme(axis.text = element_text(colour = "black", size = 14), #setrting theme
        axis.title = element_text(colour = "black", size = 14),
        legend.text = element_text(size = 14),
        legend.title = element_blank(),
        legend.position = "bottom",
        legend.box = "horizontal",
        legend.direction = "horizontal") +
  geom_point(                              #individuals
    data=data.frame(pcx.abund.G$x) %>%   rownames_to_column(var = "SampleID")%>%
      left_join(meta, by = "SampleID"),
    aes(x=PC1, y=PC2, fill=Soil_sample),
    shape=21, size=4) +
  geom_vline(xintercept = 0, linetype = 2) +   #lines-cross
  geom_hline(yintercept = 0, linetype = 2) +
  scale_fill_manual(values = c("darkgoldenrod4", "#365238"))+
  ggrepel::geom_label_repel(data = vars_choosing, aes(x=PC1, y=PC2, label= tax),
                            segment.colour = NA, box.padding = 2, fontface="italic")+
  geom_segment(data = vars_choosing, aes(x = 0, y = 0, xend = PC1, yend = PC2),
               arrow=arrow(length=unit(0.15,"cm")), #arrows and names
               alpha = 0.75, color = 'black', size= 0.6)

pca_stage_arrows_G
```



```
#pdf("fig_PCA_grainfilling.pdf", width=5, height=5)
#print(pca_stage_arrows_G)
#dev.off()
```

VII. PICRUST PLOT

Loading libraries

```
library(ComplexHeatmap)
library(tidyverse)
library(circlize)
library(viridis)
library(RColorBrewer)
library(cowplot)
```

Setting common annotations to heatmap

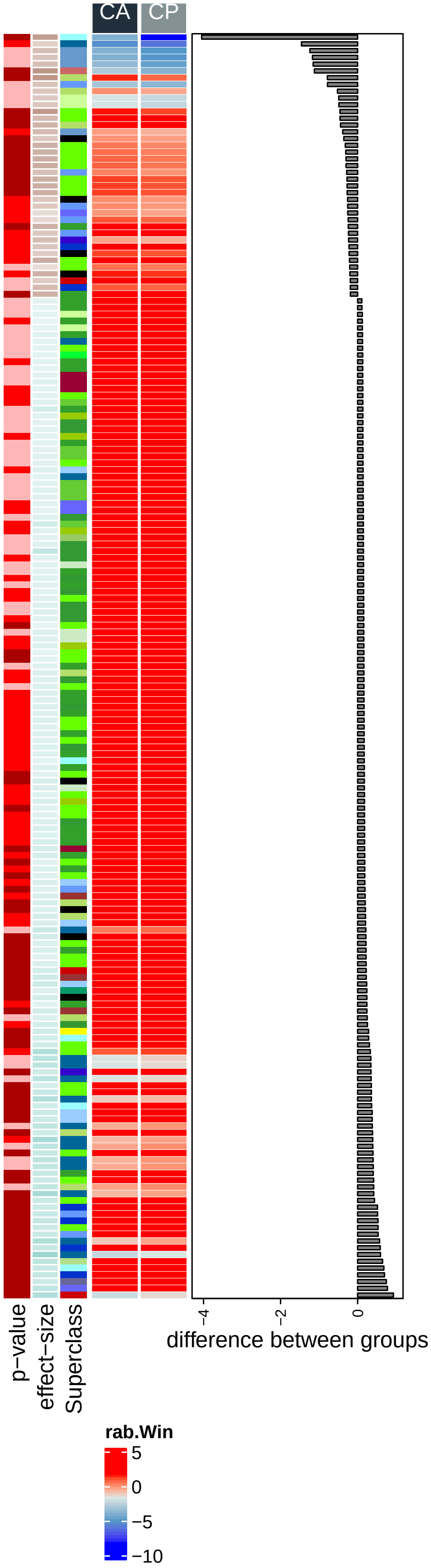
```
levels<- read_tsv( "../Data/levels.tsv")
```

```
##
## -- Column specification --------------------------------------------------------
## cols(
##   pathway = col_character(),
##   description = col_character(),
##   level1 = col_character(),
##   level2 = col_character(),
##   level3 = col_character()
## )
```

```
cols_ann <- list('Superclass' = c(
  "Alcohol Degradation"="#A6CEE3",
  "Aldehyde Degradation"="#00FFFF",
  "Amine and Polyamine Biosynthesis"="#B2DF8A",
  "Amine and Polyamine Degradation"="#3300CC",
  "Amino Acid Biosynthesis"="#33A02C",
  "Amino Acid Degradation"="#99FFFF",
  "Aminoacyl-tRNA Charging"="#99CC66",
  "Aromatic Compound Degradation"="#006699",
  "C1 Compound Utilization and Assimilation"="#6699CC",
  "Carbohydrate Biosynthesis"="#B3DE69",
  "Carbohydrate Degradation"="#6699FF",
  "Carboxylate Degradation"="#0033CC",
  "Cell Structure Biosynthesis"="#CCEBC5",
  "Cofactor, Carrier, and Vitamin Biosynthesis"="#66FF00",
  "Cofactor, Prosthetic Group, Electron Carrier Degradation"="#00CCFF",
  "Degradation/Utilization/Assimilation"="#666699",
  "Fatty Acid and Lipid Biosynthesis"="#66CC33",
  "Fatty Acid and Lipid Degradation"="#000666",
  "Fermentation"="#CC0000",
  "Glycolysis"="#993333",
  "Inorganic Nutrient Metabolism"="#6666FF",
  "Metabolic Regulator Biosynthesis"="#669933",
  "Nucleic Acid Processing"="#FFFF00",
  "Nucleoside and Nucleotide Biosynthesis"="#339933",
  "Nucleoside and Nucleotide Degradation"="#99CCFF",
  "Other"="#000000",
  "Other Biosynthesis"="#069966",
  "Pentose Phosphate Pathways"="#FF6666",
  "Polyprenyl Biosynthesis"="#00FF33",
  "Respiration"="#CC6666",
  "Secondary Metabolite Biosynthesis"="#99CC00",
  "Secondary Metabolite Degradation"="#66CCCC",
  "TCA cycle"="#990033",
  "Tetrapyrrole Biosynthesis"="#CCFF99"))

cols_pvalue <- list('p-value' = c("<0.001" = '#AB0000',
                                  "<0.01" = '#FF0000',
                                  "<0.05"="#FFB6B6"))

effect_col_fun =colorRamp2(c(-1.5, 0, 1.5), c(
  "lightsalmon4", "white", "lightseagreen"))
```

Treatment Picrust

```
aldex_all_dif<- read_tsv( "../Data/aldex_Treatment_picrust.tsv")

annotation_heatmap<- aldex_all_dif%>% left_join(
  levels, by = c("Feature.ID"="pathway"))%>% dplyr::select(
  level2, Feature.ID, p.Value, effect, diff.btw) %>% mutate_at(c(
    3), funs(p.value = case_when(
    . <= 0.001 ~ "<0.001",
    . >  0.001 & .  <= 0.01 ~ "<0.01",
    . >  0.01 & .  <= 0.05 ~ "<0.05")))%>%arrange(
      diff.btw)%>%column_to_rownames(var = "Feature.ID")

data_heatmap<- aldex_all_dif %>% arrange(diff.btw)%>%column_to_rownames(
  var = "Feature.ID")%>%dplyr::select(
    rab.win.CA, rab.win.CP, diff.btw) %>% arrange(diff.btw)

color_heatmap= colorRamp2(seq(min(data_heatmap), max(data_heatmap), length = 5), c(
  "#0000FF","#5499C7", "#DAE7E4",  "red", "#FF0000"))

colAnn <- HeatmapAnnotation(Superclass = annotation_heatmap$level2,
                            which = 'row',
                            col = cols_ann,
                            show_legend = F)

annP2 = HeatmapAnnotation("p-value" = annotation_heatmap$p.value,
                          which = "row", col = cols_pvalue,
                          show_legend = F)

annEffect = HeatmapAnnotation("effect-size" = annotation_heatmap$effect,
                              which = "row", col = list(
                              "effect-size" = effect_col_fun),
                              show_legend = F,
                              gp = gpar(col = "white"))

bardif= rowAnnotation(
  "difference between groups" = anno_barplot(
    annotation_heatmap$diff.btw, width = unit(4, "cm")))



ht5<-ComplexHeatmap::Heatmap(
  data_heatmap[-3],
  row_dend_reorder = F, col = color_heatmap,
  width = ncol(data_heatmap)*unit(0.6, "cm"),
  height = ncol(data_heatmap)*unit(8, "cm"),
  left_annotation =  c(annP2, annEffect, colAnn),
  heatmap_legend_param = list(direction = "vertical" ),
  right_annotation = c(bardif),
  cluster_column_slices = FALSE,
  column_split = rep(c("CA", "CP")),
  cluster_rows = F,
  column_km = 1, column_title_gp = gpar(
  fill = c("#212F3D", "#839192" ), col="white"),
  border = F, column_gap = unit(0.5, "mm"),
  row_dend_side = "left",row_names_side = "right",show_row_names = F ,
  rect_gp = gpar(col = "white", lwd = 0.2), row_names_gp = gpar(
  fontface ="italic", fontsize=10),
  cluster_columns = F,
  show_column_names = F, name = "rab.Win")

#ht5

ht5.2<-draw(ht5, heatmap_legend_side = "bottom")
```

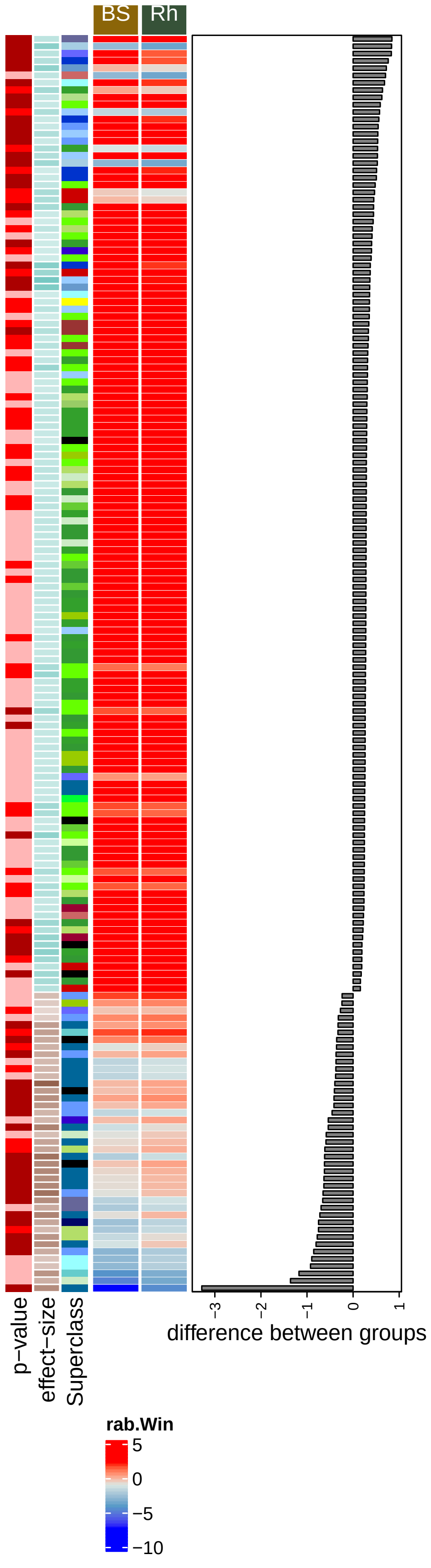

CA
CP
p−value
effect−size
Superclass
-4
-2
0
difference between groups
rab.Win
5
0
−5
−10

```
#pdf("fig_picrust_TREATMENT2.pdf", width=7, height=20)
#print(ht5.2)
#dev.off()

#pdf("fig_picrust_TREATMENT.pdf", width=10, height=10)
#print(ht5)
#dev.off()
```

Soil Picrust

```
aldex_all_dif<- read_tsv( "../Data/aldex_Soil_picrust.tsv")

annotation_heatmap<- aldex_all_dif%>% left_join(levels, by = c(
  "Feature.ID"="pathway"))%>% dplyr::select(
  level2, Feature.ID, p.Value, effect, diff.btw, rab.win.Rh, rab.win.Bs )%>% mutate_at(
    c(3), funs(p.value = case_when(
    . <= 0.001 ~ "<0.001",
    . >  0.001 & .  <= 0.01 ~ "<0.01",
    . >  0.01 & .  <= 0.05 ~ "<0.05")))%>% mutate(
      diff.btw2 = diff.btw*-1, effect2 = effect*-1 ) %>% arrange(
      diff.btw2)%>%column_to_rownames(var = "Feature.ID")

data_heatmap<- annotation_heatmap%>%dplyr::select(
  rab.win.Bs, rab.win.Rh, diff.btw2 ) %>% arrange(
    diff.btw2)

color_heatmap= colorRamp2(
  seq(min(data_heatmap), max(data_heatmap),
  length = 5), c("#0000FF","#5499C7", "#DAE7E4",  "red", "#FF0000"))


colAnn <- HeatmapAnnotation(Superclass = annotation_heatmap$level2,
                            which = 'row',
                            col = cols_ann,
                            show_legend = F)

annP2 = HeatmapAnnotation("p-value" = annotation_heatmap$p.value,
                          which = "row", col = cols_pvalue,
                          show_legend = F)

annEffect = HeatmapAnnotation("effect-size" = annotation_heatmap$effect,
                              which = "row", col = list(
                              "effect-size" = effect_col_fun),
                              show_legend = F,
                              gp = gpar(col = "white"))

bardif= rowAnnotation(
  "difference between groups" = anno_barplot(
    annotation_heatmap$diff.btw, width = unit(4, "cm")))
```

```
ht4<-ComplexHeatmap::Heatmap(
  data_heatmap[-3],  row_dend_reorder = F, col = color_heatmap,
  width = ncol(data_heatmap)*unit(0.6, "cm"),
  height = ncol(data_heatmap)*unit(8, "cm"),
  left_annotation =  c(annP2, annEffect, colAnn),
  heatmap_legend_param = list(direction = "vertical" ),
  right_annotation = c(bardif),
  cluster_column_slices = FALSE,
  column_split = rep(c("BS", "Rh")),
  show_heatmap_legend = T,
  cluster_rows = F,
  column_km = 1, column_title_gp = gpar(
  fill = c("darkgoldenrod4", "#365238" ), col="white"),
  border = F, column_gap = unit(0.5, "mm"),
  row_dend_side = "left",row_names_side = "right",show_row_names = F ,
  rect_gp = gpar(col = "white", lwd = 0.2), row_names_gp = gpar(
  fontface ="italic", fontsize=10),
  cluster_columns = F,
  show_column_names = F, name = "rab.Win")

ht4.2<-draw(ht4, heatmap_legend_side = "bottom")
```

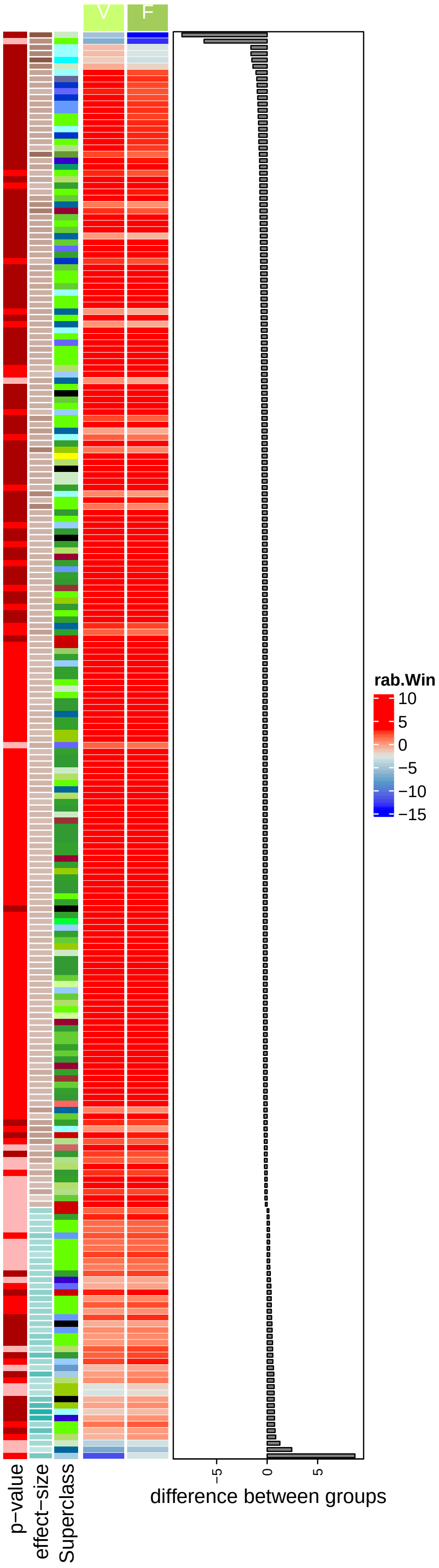

BS
Rh
p−value
effect−size
Superclass
−3
−2
−1
0
1
difference between groups
rab.Win
5
0
−5
−10

```
#pdf("fig_picrust_soil2.pdf", width=7, height=20)
#print(ht4.2)
#dev.off()

#pdf("fig_picrust_soil.pdf", width=10, height=10)
#print(ht4)
#dev.off()
```

Stage Picrust

```
# VvsF
aldex_all_dif<- read_tsv( "../Data/aldex_Stage_vvsf_picrust.tsv")


#contruct heatmap
annotation_heatmap<- aldex_all_dif%>% left_join(
  levels, by = c("Feature.ID"="pathway"))%>% dplyr::select(
  level2, Feature.ID, p.Value, effect, diff.btw) %>% mutate_at(c(3), funs(
    p.value = case_when(
    . <= 0.001 ~ "<0.001",
    . >  0.001 & .  <= 0.01 ~ "<0.01",
    . >  0.01 & .  <= 0.05 ~ "<0.05")))%>%arrange(
      diff.btw)%>%column_to_rownames(var = "Feature.ID")

data_heatmap<- aldex_all_dif %>% arrange(
  diff.btw)%>%column_to_rownames(
var = "Feature.ID")%>%dplyr::select(
  rab.win.0, rab.win.1, diff.btw) %>% rename(
  Ve=rab.win.0 , Fl=rab.win.1) %>% arrange(diff.btw)

colAnn <- HeatmapAnnotation(
  Superclass = annotation_heatmap$level2,
                            which = 'row',
                            col = cols_ann,
                            show_legend = F)


annP2 = HeatmapAnnotation("p-value" = annotation_heatmap$p.value,
                          which = "row", col = cols_pvalue,
                          show_legend = F)


#effect annotation

annEffect = HeatmapAnnotation(
  "effect-size" = annotation_heatmap$effect,
   which = "row", col = list("effect-size" = effect_col_fun),
   show_legend  =F,
   gp = gpar(col = "white"))

#barplot annotation
bardif= rowAnnotation(
  "difference between groups" = anno_barplot(
    annotation_heatmap$diff.btw, width = unit(4, "cm")))



color_heatmap= colorRamp2(
  seq(min(data_heatmap), max(
    data_heatmap), length = 5), c(
      "#0000FF","#5499C7", "#DAE7E4",  "red", "#FF0000"))

htVvsF<-  ComplexHeatmap::Heatmap(
  as.matrix(data_heatmap[-3]), col = color_heatmap, row_dend_reorder = F,
  width = ncol(data_heatmap)*unit(0.6, "cm"),
  height = ncol(data_heatmap)*unit(10, "cm"),
  left_annotation =  c(annP2,annEffect, colAnn),
  heatmap_legend_param = list(direction = "vertical" ),
  right_annotation = c(bardif),
  column_split = factor(rep(c("V", "F")), levels = c("V", "F")),
  cluster_rows = F,
  column_km = 1,
  column_title_gp = gpar(fill = c(
  "darkolivegreen1","darkolivegreen3"), col="white"),
   border = F, column_gap = unit(0.5, "mm"), row_dend_side = "left",
  row_names_side = "right",show_row_names = F ,
  rect_gp = gpar(col = "white", lwd = 0.2), row_names_gp = gpar(
  fontface ="italic", fontsize=10),
  show_column_names = F, name = "rab.Win",
  cluster_columns = F,
  cluster_column_slices = F)

htVvsF
```

Vegetative vs Flowering

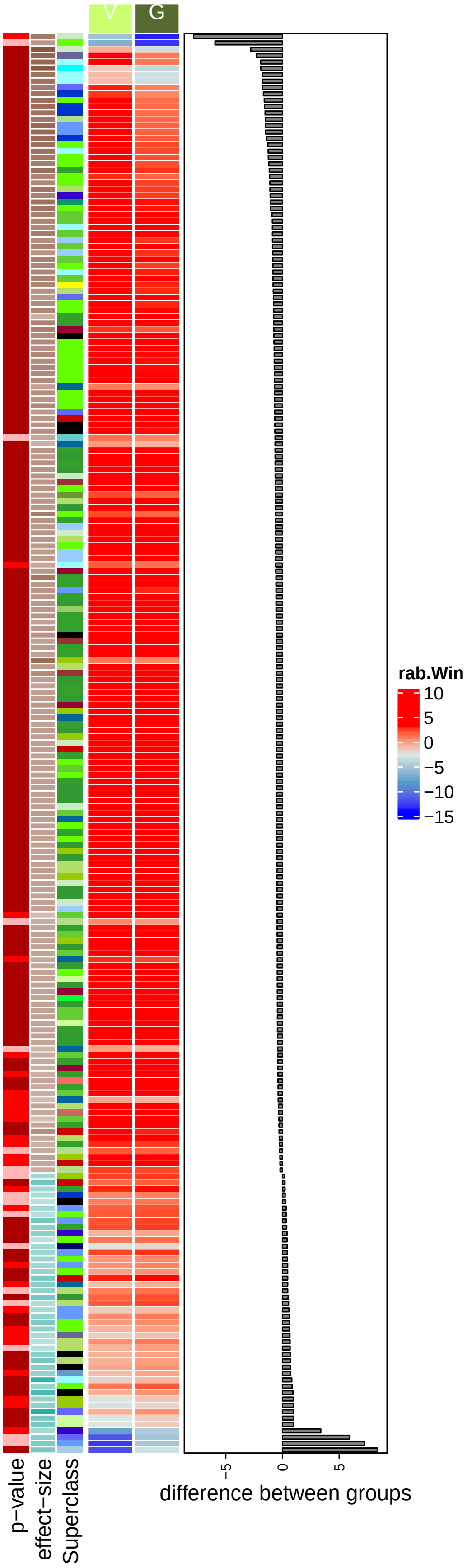

V
F
rab.Win
10
5
0
−5
−10
−15
p−value
effect−size
Superclass
-5
0
5
difference between groups

```r
#pdf("fig_picrust_VvsF.pdf", width=7, height=20)
#print(htVvsF)
#dev.off()
```

```r
# VvsF
aldex_all_dif<- read_tsv( "../Data/aldex_Stage_vvsg_picrust.tsv")


#construc heatmap
annotation_heatmap<- aldex_all_dif%>% left_join(
  levels, by = c("Feature.ID"="pathway"))%>% dplyr::select(
  level2, Feature.ID, p.Value, effect, diff.btw) %>% mutate_at(
    c(3), funs(p.value = case_when(
    . <= 0.001 ~ "<0.001",
    . >  0.001 & .  <= 0.01 ~ "<0.01",
    . >  0.01 & .  <= 0.05 ~ "<0.05")))%>%arrange(
      diff.btw)%>%column_to_rownames(var = "Feature.ID")

data_heatmap<- aldex_all_dif %>% arrange(
  diff.btw)%>%column_to_rownames(
  var = "Feature.ID")%>%dplyr::select(
    rab.win.0, rab.win.1) %>%  rename(
  Ve= rab.win.0, Gr= rab.win.1)

colAnn <- HeatmapAnnotation(
  Superclass = annotation_heatmap$level2,
  which = 'row',
  col = cols_ann,
  show_legend = F)

cols_pvalue <- list(
  'p-value' = c("<0.001" = '#AB0000',
  "<0.01" = '#FF0000',
  "<0.05"="#FFB6B6"))

annP2 = HeatmapAnnotation(
  "p-value" = annotation_heatmap$p.value,
   which = "row", col = cols_pvalue,
   show_legend = F)


#effect annotation
annEffect = HeatmapAnnotation(
  "effect-size" = annotation_heatmap$effect,
  which = "row", col = list(
  "effect-size" = effect_col_fun),
  show_legend  =F,
  gp = gpar(col = "white"))

bardif= rowAnnotation(
  "difference between groups" = anno_barplot(
    annotation_heatmap$diff.btw, width = unit(4, "cm")))
```

```r
htVvsG<-ComplexHeatmap::Heatmap(
  data_heatmap, col = color_heatmap, row_dend_reorder = F,
  width = ncol(data_heatmap)*unit(0.9, "cm"),
  height = ncol(data_heatmap)*unit(14, "cm"),
  left_annotation =  c(annP2, annEffect, colAnn),
  heatmap_legend_param = list(direction = "vertical" ),
  right_annotation = c(bardif),
  cluster_column_slices = FALSE,
  column_split = factor(rep(c("V", "G")), levels = c("V", "G")),
  cluster_rows = F,show_heatmap_legend = T,
  column_km = 1, column_title_gp = gpar(fill = c(
  "darkolivegreen1","darkolivegreen"), col="white"),
  border = F, column_gap = unit(0.5, "mm"),
  row_dend_side = "left",row_names_side = "right",show_row_names = F ,
  rect_gp = gpar(col = "white", lwd = 0.2),
  row_names_gp = gpar(fontface ="italic", fontsize=10),
  cluster_columns = F,
  show_column_names = F, name = "rab.Win")


htVvsG
```

Vegetative vs Grainfilling

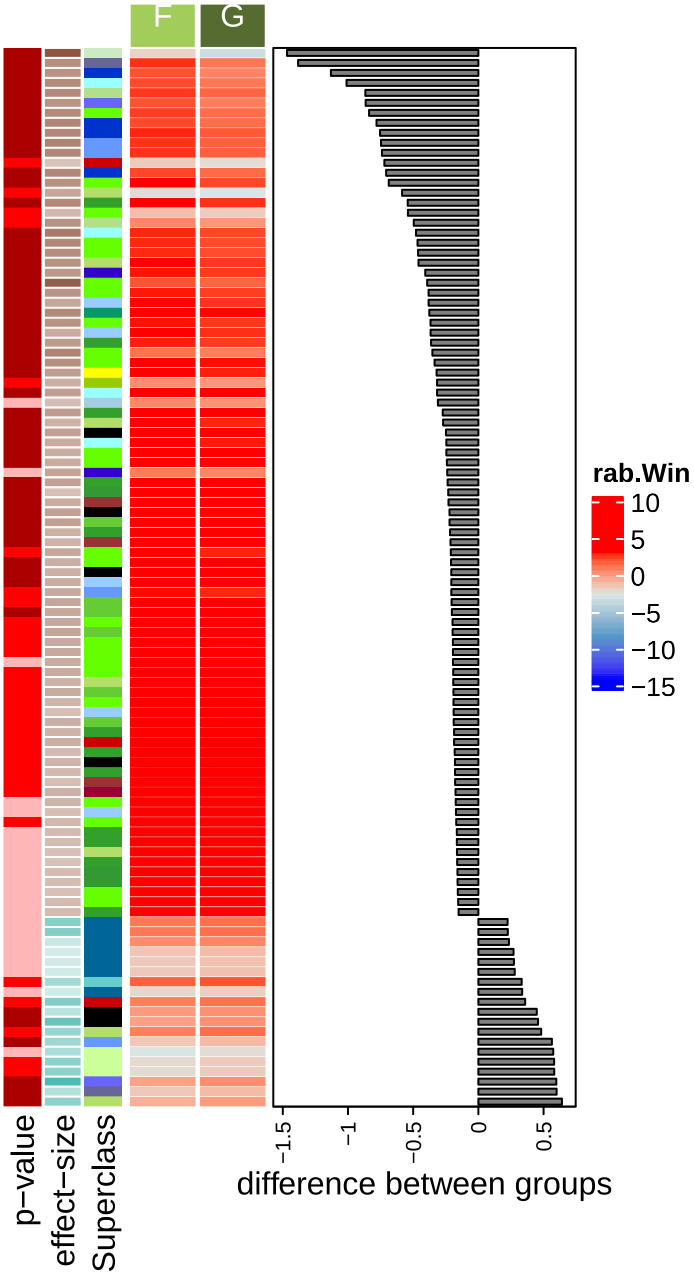

V
G
rab.Win
10
5
0
−5
−10
−15
−5
0
5
difference between groups
p−value
effect−size
Superclass

```
#pdf("fig_picrust_VvsG.pdf", width=7, height=20)
#print(htVvsG)
#dev.off()
```

```
aldex_all_dif<- read_tsv( "../Data/aldex_Stage_fvsg_picrust.tsv")


#construc heatmap
annotation_heatmap<- aldex_all_dif%>% left_join(
  levels, by = c("Feature.ID"="pathway"))%>% dplyr::select(
  level2, Feature.ID, p.Value, effect, diff.btw) %>% mutate_at(
  c(3), funs(p.value = case_when(
  . <= 0.001 ~ "<0.001",
  . >  0.001 & .  <= 0.01 ~ "<0.01",
  . >  0.01 & .  <= 0.05 ~ "<0.05")))%>%arrange(
  diff.btw)%>%column_to_rownames(var = "Feature.ID")

data_heatmap<- aldex_all_dif %>% arrange(
  diff.btw)%>%column_to_rownames(
  var = "Feature.ID")%>%dplyr::select(
  rab.win.0, rab.win.1) %>% rename(
  Fl = rab.win.0, Gr= rab.win.1)

colAnn <- HeatmapAnnotation(
  Superclass = annotation_heatmap$level2,
  which = 'row',
  col = cols_ann,
  show_legend = F)

cols_pvalue <- list(
  'p-value' = c("<0.001" = '#AB0000',
  "<0.01" = '#FF0000',
  "<0.05"="#FFB6B6"))

annP2 = HeatmapAnnotation(
  "p-value" = annotation_heatmap$p.value,
   which = "row", col = cols_pvalue,
   show_legend = F)

#effect annotation

annEffect = HeatmapAnnotation(
  "effect-size" = annotation_heatmap$effect,
   which = "row", col = list("effect-size" = effect_col_fun),
   show_legend  =F,
   gp = gpar(col = "white"))

bardif= rowAnnotation(
  "difference between groups" = anno_barplot(
    annotation_heatmap$diff.btw, width = unit(4, "cm")))
```

```r
htFvsG<-ComplexHeatmap::Heatmap(
  data_heatmap, col = color_heatmap, row_dend_reorder = F,
  width = ncol(data_heatmap)*unit(0.9, "cm"),
  height = ncol(data_heatmap)*unit(7, "cm"),
  left_annotation =  c(annP2, annEffect, colAnn),
  heatmap_legend_param = list(direction = "vertical" ),
  right_annotation = c(bardif),
  column_split = rep(c("F", "G")),
  cluster_rows = F, show_heatmap_legend = T,
  cluster_column_slices = F,
  column_km = 1, column_title_gp = gpar(
  fill = c("darkolivegreen3","darkolivegreen" ), col="white"),
  border = F, column_gap = unit(0.5, "mm"),
  cluster_columns = F,
  row_dend_side = "left",row_names_side = "right",show_row_names = F ,
  rect_gp = gpar(col = "white", lwd = 0.2), row_names_gp = gpar(
  fontface ="italic", fontsize=10),show_column_names = F, name = "rab.Win")

htFvsG
```

Flowering vs Grainfilling



```
#pdf("fig_picrust_FvsG.pdf", width=7, height=20)
#print(htFvsG)
#dev.off()
```

VIII. PICRUST2 PLOT

Loading libraries

```
library(ComplexHeatmap)
library(tidyverse)
library(circlize)
library(viridis)
library(RColorBrewer)
library(cowplot)
```

Loadings files

```
aldex_all_dif_soil<- read_tsv( "../Data/aldex_Soil_picrust.tsv") %>% arrange(diff.btw)
aldex_all_dif_Treatment<- read_tsv( "../Data/aldex_Treatment_picrust.tsv")%>% arrange(diff.btw)
aldex_all_dif_Stage_vvsf<- read_tsv( "../Data/aldex_Stage_vvsf_picrust.tsv")%>% arrange(diff.btw)
aldex_all_dif_Stage_vvsg<- read_tsv( "../Data/aldex_Stage_vvsg_picrust.tsv")%>% arrange(diff.btw)
aldex_all_dif_Stage_fvsg<- read_tsv( "../Data/aldex_Stage_fvsg_picrust.tsv")%>% arrange(diff.btw)
```

Formatting files

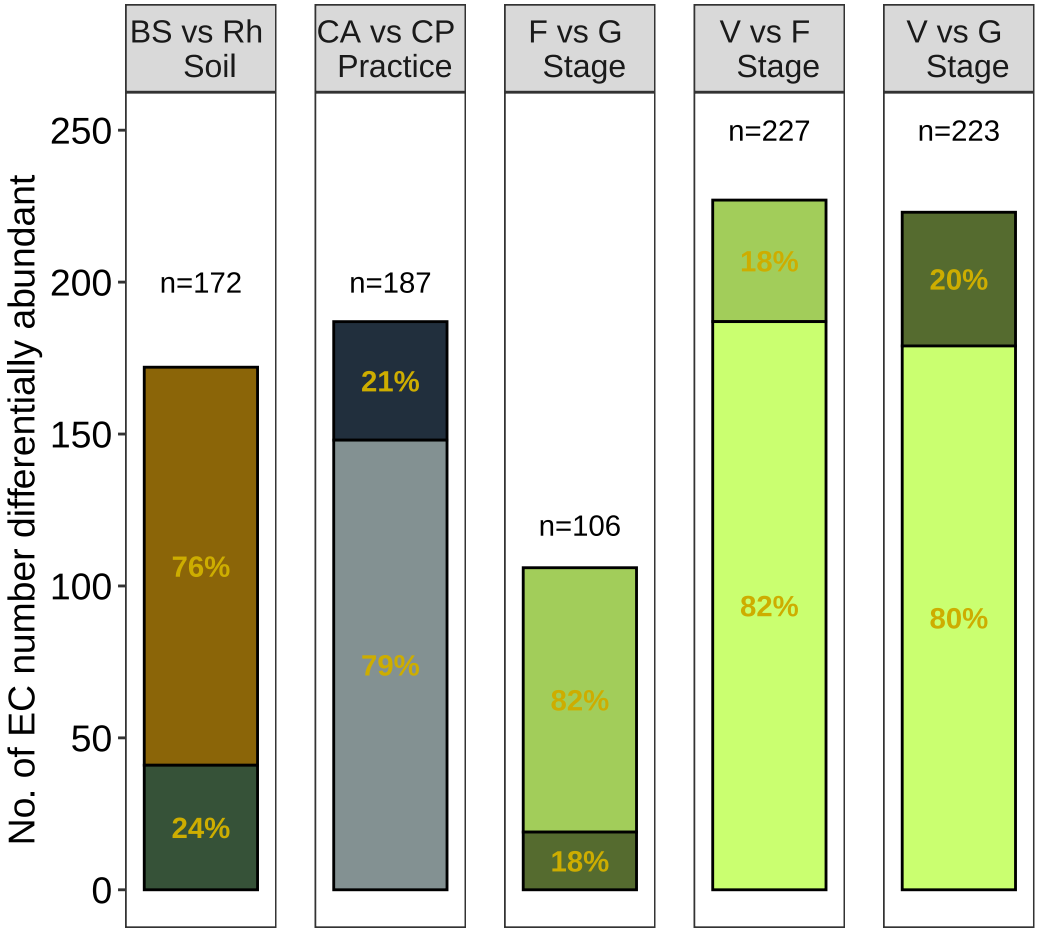
```
a<-aldex_all_dif_soil %>%  mutate(Dif = case_when(
  diff.btw < 0 ~ "RH",
  diff.btw > 0  ~ "BS")) %>% group_by(Dif) %>%
  summarise(n = n()) %>%
  mutate(freq = round(n / sum(n)*100)) %>% mutate(type = "BS vs Rh \n  Soil")

b<-aldex_all_dif_Treatment %>%  mutate(Dif = case_when(
  diff.btw < 0 ~ "CA",
  diff.btw > 0  ~ "CP")) %>% group_by(Dif) %>%
  summarise(n = n()) %>%
  mutate(freq = round(n / sum(n)*100))%>% mutate(type = "CA vs CP \n Practice")

c<-aldex_all_dif_Stage_vvsf %>%  mutate(Dif = case_when(
  diff.btw < 0 ~ "V",
  diff.btw > 0  ~ "F")) %>% group_by(Dif) %>%
  summarise(n = n()) %>%
  mutate(freq = round(n / sum(n)*100))%>% mutate(type = "V vs F \n  Stage")

d<-aldex_all_dif_Stage_vvsg %>%  mutate(Dif = case_when(
  diff.btw < 0 ~ "V",
  diff.btw > 0  ~ "G")) %>% group_by(Dif) %>%
  summarise(n = n()) %>%
  mutate(freq = round(n / sum(n)*100))%>% mutate(type = "V vs G \n  Stage")

e<-aldex_all_dif_Stage_fvsg%>%  mutate(Dif = case_when(
  diff.btw < 0 ~ "F",
  diff.btw > 0  ~ "G")) %>% group_by(Dif) %>%
  summarise(n = n()) %>%
  mutate(freq = round(n / sum(n)*100))%>% mutate(type = "F vs G \n Stage")

#joining all files
graph<- rbind(a,b,c,d,e) %>%mutate(
  freq2= paste(freq,"%", sep = ""))

#setting labels (sum of n by type)

label2<- paste("n=",graph$n[1]+graph$n[2], sep = "")
label1<- paste("n=",graph$n[3]+graph$n[4], sep = "")
label5<- paste("n=",graph$n[5]+graph$n[6], sep = "")
```

```
label4<- paste("n=",graph$n[7]+graph$n[8], sep = "")
label3<- paste("n=",graph$n[9]+graph$n[10], sep = "")


head(graph)
```

```
## # A tibble: 6 x 5
##   Dif       n  freq type                   freq2
##   <chr> <int> <dbl> <chr>                  <chr>
## 1 BS      131    76 "BS vs Rh \n  Soil"    76%
## 2 RH       41    24 "BS vs Rh \n  Soil"    24%
## 3 CA       39    21 "CA vs CP \n Practice" 21%
## 4 CP      148    79 "CA vs CP \n Practice" 79%
## 5 F        40    18 "V vs F \n  Stage"     18%
## 6 V       187    82 "V vs F \n  Stage"     82%
```

Barplot

```
#annotation to facets

ann_text<-data.frame(type=c("BS vs Rh \n  Soil", "CA vs CP \n Practice",
                            "F vs G \n Stage","V vs F \n  Stage", "V vs G \n  Stage"),
                     n=c(200,200,120, 250, 250),
                     Dif=c("BS","CA","G", "F", "V"),
                     label=c(label2, label1, label3, label5, label4))
#plot

graphs2=graph %>%  ggplot(aes(x = type, y = n, fill = Dif))+ geom_bar(stat = "identity",color="black")+
  ylab("No. of EC number differentially abundant")+
  geom_text(data = graph, aes(label=freq2),position = position_stack(vjust = 0.5),  size=4, color = "gol
  geom_text(data = ann_text,label=ann_text$label)  + theme_bw()+
  theme(panel.spacing=unit(1,"lines"),
        strip.text.x = element_text(size = 12),
        axis.text.y =  element_text(colour = "black", size = 14),
        axis.text.x = element_blank(),
        axis.title.x = element_blank(),
        axis.ticks.x=element_blank(),
        axis.title.y = element_text(size = 14),
        legend.title = element_text(size = 12),
        legend.text = element_text(size=12),
        panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        legend.direction = "vertical" ,
        legend.position = "none")+scale_fill_manual(
          values = c("darkgoldenrod4",
                     "#212F3D", "#839192","darkolivegreen3",
                     "darkolivegreen",
                     "#365238", "darkolivegreen1"))
   graphs2
```



```
#pdf("fig_picrust_ECnumber.pdf", width=5.5, height=5)
#print(graphs2)
#dev.off()
```

Bolyen, Evan, Jai Ram Rideout, Matthew R. Dillon, Nicholas A. Bokulich, Christian C. Abnet, Gabriel A. Al-Ghalith, Harriet Alexander, et al. 2019. "Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2." *Nature Biotechnology* 37 (8): 852–57. https://doi.org/10.1038/s41587-019-0209-9.

Douglas, Gavin M., Vincent J. Maffei, Jesse R. Zaneveld, Svetlana N. Yurgel, James R. Brown, Christopher M. Taylor, Curtis Huttenhower, and Morgan G. I. Langille. 2020. "PICRUSt2 for Prediction of Metagenome Functions." *Nature Biotechnology* 38 (6): 685–88. https://doi.org/10.1038/s41587-020-0548-6.