TB-Profiling Analysis

Geoffrey Olweny

2024-11-18

Contents

Install necessary packages required

These are packages to be used in the analysis.

```
# List of CRAN packages
cran_packages <- c(
  "ggplot2", "pgirmess", "reshape", "dplyr", "tidyverse", "rstatix",
  "ggpubr", "cowplot", "RColorBrewer", "fs", "vegan", "ggsignif", "ggtreeExtra",
  "ggstar", "ggalluvial", "ggtree", "patchwork", "randomForest",
  "caret", "ROCR", "e1071", "gridExtra", "fastDummies", "here", "pROC"
)
# List of Bioconductor packages
bioc_packages <- c(
  "metadeconfoundR", "DirichletMultinomial", "phyloseq", "edgeR", "MicrobiotaProcess"
)

# Function to check if a package is installed, and if not, install it
install_if_missing_cran <- function(pkg) {
  if (!requireNamespace(pkg, quietly = TRUE)) {
    install.packages(pkg, dependencies = TRUE)
  }
}

# Function to check if a Bioconductor package is installed, and if not, install it
install_if_missing_bioc <- function(pkg) {
  if (!requireNamespace(pkg, quietly = TRUE)) {
    BiocManager::install(pkg)
  }
}

# Apply the function to each CRAN package
lapply(cran_packages, install_if_missing_cran)

# Apply the function to each Bioconductor package
lapply(bioc_packages, install_if_missing_bioc)

# Install EasyStat from Git
if (!requireNamespace("EasyStat", quietly = TRUE)) {
  remotes::install_git('https://gitee.com/wentaomicro/EasyStat')
}

# Install ggClusterNet from GitHub
if (!requireNamespace("ggClusterNet", quietly = TRUE)) {
  remotes::install_github("taowenmicro/ggClusterNet")
```

```
}

# Load all the packages
all_packages <- c(cran_packages, bioc_packages, "EasyStat", "ggClusterNet")
lapply(all_packages, library, character.only = TRUE)
```

Load the necessary packages

These are to be used during analysis.

```
# Load required libraries
library(metadeconfoundR)
library(DirichletMultinomial)
library(phyloseq)
library(ggplot2)
library(pgirmess)
library(reshape)
library(dplyr)
library(tidyverse)
library(rstatix)
library(ggpubr)
library(cowplot)
library(fs)
library(vegan)
library(ggClusterNet)
library(EasyStat)
library(ggsignif)
library(ggtreeExtra)
library(ggstar)
library(ggalluvial)
library(ggtree)
library(patchwork)
library(edgeR)
library(randomForest)
library(caret)
library(ROCR)
library(e1071)
library(MicrobiotaProcess)
library(gridExtra)
library(fastDummies)
library(RColorBrewer)
library(here)

sessionInfo()
```

Reading in the Files

These are input files required for the protocol.

```r
# Load OTU counts files
Genexpert_otu_df <- read.table("data/Genexpert_OTU.txt", header = TRUE, row.names = 1)
MtbInfectionStatus_otu_df <- read.table("data/MtbInfectionStatus_OTU.txt", header = TRUE, row.names = 1
# Load taxonomy table
tax_df <- read.table("data/Tax.txt", header = TRUE, sep = "\t", row.names = 1, check.names = FALSE)
# Replace all "?" values in tax_df with "Unknown"
tax_df[tax_df == "?"] <- "Unknown"
# Load metadata files
Genexpert_metadata_df <- read.csv(file = "data/Genexpert_metadata.csv", header = TRUE, row.names = 1)
MtbInfectionStatus_metadata_df <- read.csv(file = "data/MtbInfectionStatus_metadata.csv", header = TRUE
```

Inspect files

```r
# Inspect the first few rows of Genexpert OTU counts
head(Genexpert_otu_df)
#>      P01.A01.HLM069 P01.A02.HLM002 P01.A03.HLM038 P01.A04.HLM091 P01.A05.HLM061
#> OTU1           3535          29248           2826           1357          11970
#> OTU2           4420          27781           2856           2034          12041
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5           9383            185            428           6681            749
#> OTU6          15765            264            268           2504            706
#>      P01.A06.HLM094 P01.A07.HLM102 P01.A08.HLM056 P01.A09.HLM053 P01.A10.HLM019
#> OTU1           5904           5920           4279          11938          16310
#> OTU2           5417           5628           4227          11667          15288
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5           3587            206           4768           1778           1616
#> OTU6           2915             49           2194           1789           4876
#>      P01.A11.HLM137 P01.A12.HLM131 P01.B01.HLM062 P01.B02.HLM064 P01.B03.HLM037
#> OTU1           6244           8808           1805          12257           2761
#> OTU2           6404           9541           1705          11729           2663
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5           4518             25           1364            626            303
#> OTU6           1519           5310          15212             99            190
#>      P01.B04.HLM089 P01.B05.HLM107 P01.B06.HLM097 P01.B07.HLM098 P01.B08.HLM106
#> OTU1           1992          20050           8446           3482            391
#> OTU2           1918          20003           8019           3801            407
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5           1427           5259            544           2962            109
#> OTU6           1513           8138            712           1193             31
#>      P01.B09.HLM054 P01.B10.HLM020 P01.B11.HLM136 P01.B12.HLM122 P01.C01.HLM080
#> OTU1           1394          29611           9606           5960           1994
#> OTU2           1383          28680           8337           5348           1659
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5           4015            524            803            275           6881
#> OTU6           4596            137            106             56           9915
#>      P01.C02.HLM044 P01.C03.HLM045 P01.C04.HLM074 P01.C05.HLM114 P01.C06.HLM084
```

```
#> OTU1           5927           2344           3077           6969           5311
#> OTU2           5441           2197           2707           6727           5323
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5          12482            505           1143           1521          12162
#> OTU6          14380           1087           3335           1141           6206
#>      P01.C07.HLM104 P01.C08.HLM096 P01.C09.HLM035 P01.C10.HLM021 P01.C11.HLM117
#> OTU1           9648           3421          10557           9749           7363
#> OTU2           9680           3252          10220           8320           6913
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5            892            715           2895            379           3954
#> OTU6            409            584           3472            327           1042
#>      P01.C12.HLM128 P01.D01.HLM079 P01.D02.HLM043 P01.D03.HLM025 P01.D04.HLM073
#> OTU1           3212           4528           6698           5463          10157
#> OTU2           3085           4393           6366           5463           9607
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5           2891             23            191           2169           6722
#> OTU6            504             79            232           1734           4159
#>      P01.D05.HLM090 P01.D06.HLM093 P01.D07.HLM105 P01.D08.HLM086 P01.D09.HLM055
#> OTU1           2113           4549           3833           4283          11894
#> OTU2           2012           4272           4645           3965          11934
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5            699           1705           1681            771           4302
#> OTU6           1756            148           1843            646           3561
#>      P01.D10.HLM022 P01.D11.HLM101 P01.D12.HLM135 P01.E01.HLM065 P01.E02.HLM042
#> OTU1           4232           8691           2270           2412          11017
#> OTU2           4009           7372           2124           2355          11271
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5           1186            911            204           1757           6181
#> OTU6           1057            238             75           4673           3215
#>      P01.E03.HLM049 P01.E05.HLM095 P01.E06.HLM100 P01.E07.HLM103 P01.E08.HLM112
#> OTU1           2504           3529          11085          13691           5748
#> OTU2           3111           4889          10099          13972           5486
#> OTU3              0              0              0              0              0
#> OTU4              1              0              0              0              0
#> OTU5            841          20327            470          12167            734
#> OTU6           8724          11277            870           2473            271
#>      P01.E09.HLM057 P01.E10.HLM023 P01.E11.HLM120 P01.E12.HLM138 P01.F01.HLM066
#> OTU1           3275           6643           5451           4505            362
#> OTU2           2989           7378          10802           4774            390
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5           1719          10219           3525           5241            910
#> OTU6           2397           8074          11704           3297            668
#>      P01.F02.HLM041 P01.F03.HLM048 P01.F05.HLM092 P01.F06.HLM081 P01.F07.HLM087
#> OTU1           6514           2970           8410           5342           3853
#> OTU2           6392           2624           9607           5229           3572
#> OTU3              0              1              0              1              0
#> OTU4              0              0              0              0              0
```

```
#> OTU5             89            508           2519             65            290
#> OTU6            883            322           5987            110           3627
#>      P01.F08.HLM050 P01.F09.HLM058 P01.F10.HLM024 P01.F11.HLM116 P01.F12.HLM140
#> OTU1           6201           4984           5744           4787           9264
#> OTU2           6133           4805           6176           4457           9750
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5            882           8251           1906            403           2061
#> OTU6           1187           1896           4528            340           1525
#>      P01.G01.HLM068 P01.G03.HLM067 P01.G04.HLM109 P01.G05.HLM083 P01.G06.HLM075
#> OTU1           4535           3191          11569           2189          15747
#> OTU2           3988           2811          10813           2108          16064
#> OTU3              0              1              0              1              0
#> OTU4              0              0              0              0              0
#> OTU5            372           3171            202           2343           1959
#> OTU6             25            964            121           5070           4084
#>      P01.G07.HLM072 P01.G08.HLM051 P01.G09.HLM059 P01.G10.HLM018 P01.G11.HLM123
#> OTU1          23475          14926           9789           3335          10761
#> OTU2          25045          14513          11781           3438          10048
#> OTU3              0              0              0              0              1
#> OTU4              0              0              0              0              0
#> OTU5           4407            522           1259           1606             13
#> OTU6           5502            369           4005           1657            311
#>      P01.G12.HLM178 P01.H01.HLM063 P01.H02.HLM039 P01.H03.HLM070 P01.H04.HLM110
#> OTU1           9978           1467          12026           6341           4345
#> OTU2          10178           1530          11612           6159           4122
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5           4046           6545           2995           1156           1965
#> OTU6           3867           5590           4097           3068           1500
#>      P01.H05.HLM082 P01.H06.HLM078 P01.H07.HLM099 P01.H08.HLM052 P01.H09.HLM029
#> OTU1           2424          10655          12290           3938          13266
#> OTU2           2381           9713          12073           3937          12806
#> OTU3              0              0              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5            358           1935           5245           3445            946
#> OTU6            665           1273           6731           3095            574
#>      P01.H10.HLM017 P01.H11.HLM124 P01.H12.HLM148 P02.A01.HLM146 P02.A02.HLM016
#> OTU1           1563           7811           5929           7118           7418
#> OTU2           1531           7212           5206           6512           7269
#> OTU3              0              1              0              0              0
#> OTU4              0              0              0              0              0
#> OTU5            648           4747            232           1418           4758
#> OTU6            149           1415            582            702            494
#>      P02.A03.HLM171 P02.A04.HLM004 P02.A05.HLM167 P02.A06.HLM172 P02.A07.HLM154
#> OTU1            262           5488          10108          12980           2583
#> OTU2             30           4924           9531          12689           2749
#> OTU3              1              0              0              0              0
#> OTU4              0              0              1              0              0
#> OTU5              1             20            981           1000            205
#> OTU6             16           1656           5955           1365            875
#>      P02.A08.HLM085 P02.A09.HLM134 P02.A10.HLM405 P02.A11.HLM413 P02.A12.HLM421
#> OTU1           4325           1563            164          13534            376
```

```
#> OTU2          4017          1430           176         13427           389
#> OTU3             0             0         25330         18023         20357
#> OTU4             0             0         22786         16339         18961
#> OTU5          4829          3447            38             1             0
#> OTU6          2790           553           146            34            85
#>      P02.B01.HLM145 P02.B02.HLM177 P02.B03.HLM032 P02.B04.HLM001 P02.B05.HLM165
#> OTU1            266          3775          12538         10678          5331
#> OTU2            239          3203          11561          9855          5204
#> OTU3              0             0              0             0             0
#> OTU4              0             0              0             0             0
#> OTU5            110           491           1056           903          2497
#> OTU6             82           662           5453          1617           817
#>      P02.B06.HLM013 P02.B07.HLM155 P02.B08.HLM003 P02.B09.HLM130 P02.B10.HLM406
#> OTU1           3882          5308          25768         12312          6874
#> OTU2           3692          5730          25621         11903          6450
#> OTU3              0             0              0             0          3394
#> OTU4              0             0              0             0          3109
#> OTU5            100          1139           4434           497             0
#> OTU6            196          1177           2407           222            58
#>      P02.B11.HLM414 P02.B12.HLM422 P02.C01.HLM143 P02.C02.HLM182 P02.C03.HLM033
#> OTU1           5550           324           3629          1377          4636
#> OTU2           5708           361           3453           935          4256
#> OTU3           1902           638              0             1             0
#> OTU4           1869           621              0             0             0
#> OTU5              0             0            315           490          2725
#> OTU6             30            83            283           678          3366
#>      P02.C04.HLM006 P02.C05.HLM129 P02.C06.HLM010 P02.C07.HLM157 P02.C08.HLM111
#> OTU1          14079          5316           7122          5123          24555
#> OTU2          13347          5437           6898          4150          24946
#> OTU3              0             0              0             0             0
#> OTU4              0             0              0             0             0
#> OTU5           1165          3756            238           594           607
#> OTU6            951          1291            282           147           342
#>      P02.C09.HLM127 P02.C10.HLM407 P02.C11.HLM415 P02.C12.HLM423 P02.D01.HLM115
#> OTU1           3263           949           2802             0          9134
#> OTU2           2864           900           2822             0          8126
#> OTU3              1          7896           1663         27594             0
#> OTU4              0          7246           1641         25516             0
#> OTU5            936             0            369             0           227
#> OTU6            598            41             41             1           537
#>      P02.D02.HLM186 P02.D03.HLM030 P02.D04.HLM173 P02.D05.HLM166 P02.D06.HLM189
#> OTU1          17092          7442          11468          9343          19794
#> OTU2          15934          7261          10746          9308          19297
#> OTU3              0             0              0             0             0
#> OTU4              0             0              1             0             0
#> OTU5           3830            94           3523           729           126
#> OTU6           2149            44           2671           370             3
#>      P02.D07.HLM158 P02.D08.HLM005 P02.D09.HLM400 P02.D10.HLM408 P02.D11.HLM416
#> OTU1          27441          7169              2           490            28
#> OTU2          25387          6632              0           443            20
#> OTU3              0             0          24451         13749         10195
#> OTU4              2             0          22516         13044          9262
#> OTU5             45          1439              0             0            20
```

```
#> OTU6            271             95              0            101             28
#>      P02.D12.HLM424 P02.E01.HLM144 P02.E02.HLM184 P02.E03.HLM014 P02.E04.HLM174
#> OTU1              9            3223            4669           13122           8730
#> OTU2              3            2912            4502           12316           7992
#> OTU3          49941               0               0               0              1
#> OTU4          46388               0               0               0              1
#> OTU5              0              67            1394             487            997
#> OTU6              1             411             203            1026            495
#>      P02.E05.HLM149 P02.E06.HLM190 P02.E07.HLM159 P02.E08.HLM132 P02.E09.HLM401
#> OTU1           8469           6610            5883           11059              0
#> OTU2          10078           6601            5622           11024              2
#> OTU3              0              0               0               0          35473
#> OTU4              0              0               0               0          32911
#> OTU5           8159           4653             121            3681              0
#> OTU6           4579           1015              72            5395              0
#>      P02.E10.HLM409 P02.E11.HLM417 P02.E12.HLM425 P02.F01.HLM179 P02.F02.HLM187
#> OTU1             76              2               1             163          23064
#> OTU2             20              1               0             177          22480
#> OTU3           3404          14098             861               0              0
#> OTU4           3296          13335             801               0              0
#> OTU5              0              1               1               8            837
#> OTU6             14              0               3               3            616
#>      P02.F03.HLM027 P02.F04.HLM175 P02.F05.HLM163 P02.F06.HLM126 P02.F07.HLM161
#> OTU1           5740           2661            5078            7113           15539
#> OTU2           5417           2670            5210            6454           15336
#> OTU3              0              0               0               0              0
#> OTU4              0              1               0               0              0
#> OTU5           1183             54            2256            2790            563
#> OTU6           1210            344            1359              84           1287
#>      P02.F08.HLM007 P02.F09.HLM402 P02.F10.HLM410 P02.F11.HLM418 P02.F12.HLM426
#> OTU1           5304            169             156            1462            303
#> OTU2           5351            169             123            1565            301
#> OTU3              0           7180             569            5554           3093
#> OTU4              0           6663             622            5022           2846
#> OTU5           2393              0               0             146             97
#> OTU6           2132             57              48              46              4
#>      P02.G01.HLM142 P02.G02.HLM188 P02.G03.HLM036 P02.G04.HLM147 P02.G05.HLM164
#> OTU1           6322           7875           12443            5244           4841
#> OTU2           6269           7410           11548            4421           4486
#> OTU3              0              0               0               1              0
#> OTU4              0              0               0               0              0
#> OTU5            388            415             136             210            313
#> OTU6            268            245             232            4124            718
#>      P02.G06.HLM141 P02.G07.HLM118 P02.G08.HLM008 P02.G09.HLM403 P02.G10.HLM411
#> OTU1           8205           8151            6264               0             78
#> OTU2           7789           9080            5792               1            100
#> OTU3              0              0               0           37534            477
#> OTU4              0              0               0           34974            488
#> OTU5           2173           8052              48               0              1
#> OTU6             73           5748              40               0            259
#>      P02.G11.HLM419 P02.G12.HLM427 P02.H01.HLM180 P02.H02.HLM176 P02.H03.HLM031
#> OTU1           3551            456             380           12289            933
#> OTU2           3401            454             352           11797            913
```

```
#> OTU3          10683          4538             0             0             0
#> OTU4          10204          4331             0             0             0
#> OTU5              0            23             6           555           342
#> OTU6            158             6            13           591           140
#>      P02.H04.HLM162 P02.H05.HLM009 P02.H06.HLM152 P02.H07.HLM119 P02.H08.HLM011
#> OTU1           6043          14173          9164          10575          1758
#> OTU2           5930          13656          8558           8710          1827
#> OTU3              0              2             1              0             0
#> OTU4              0              0             1              0             0
#> OTU5           4398           3933          2433           1728          1245
#> OTU6           2052           3379           870            657           583
#>      P02.H09.HLM404 P02.H10.HLM412 P02.H11.HLM420 P02.H12.HLM428 P03.A01.HLM429
#> OTU1              1            131            13              4           237
#> OTU2              0            180           145              1           260
#> OTU3           9189          22068          8178           6681          3386
#> OTU4           8597          20503          7712           6297          3218
#> OTU5              0              0             0              0             0
#> OTU6              0             69           410             12             6
#>      P03.B01.HLM430 P03.C01.HLM431 P03.D01.NEC
#> OTU1           1147             31           0
#> OTU2           1172             21           0
#> OTU3          17435             47           0
#> OTU4          16101             36           0
#> OTU5              0              1           0
#> OTU6              8            158           0

# Inspect the first few rows of Mtb Infection Status OTU counts
head(MtbInfectionStatus_otu_df)
#>      P01.A06.HLM094 P01.A07.HLM102 P01.A09.HLM053 P01.A11.HLM137 P01.A12.HLM131
#> OTU3              0              0              0              0             0
#> OTU4              0              0              0              0             0
#> OTU1           5904           5920          11938           6244          8808
#> OTU2           5417           5628          11667           6404          9541
#> OTU5           3587            206           1778           4518            25
#> OTU6           2915             49           1789           1519          5310
#>      P01.B10.HLM020 P01.C01.HLM080 P01.C09.HLM035 P01.D01.HLM079 P01.D04.HLM073
#> OTU3              0              0              0              0             0
#> OTU4              0              0              0              0             0
#> OTU1          29611           1994          10557           4528         10157
#> OTU2          28680           1659          10220           4393          9607
#> OTU5            524           6881           2895             23          6722
#> OTU6            137           9915           3472             79          4159
#>      P01.D06.HLM093 P01.D07.HLM105 P01.D08.HLM086 P01.E02.HLM042 P01.E06.HLM100
#> OTU3              0              0              0              0             0
#> OTU4              0              0              0              0             0
#> OTU1           4549           3833           4283          11017         11085
#> OTU2           4272           4645           3965          11271         10099
#> OTU5           1705           1681            771           6181           470
#> OTU6            148           1843            646           3215           870
#>      P01.E07.HLM103 P01.E10.HLM023 P01.E12.HLM138 P01.F01.HLM066 P01.F06.HLM081
#> OTU3              0              0              0              0             1
#> OTU4              0              0              0              0             0
#> OTU1          13691           6643           4505            362          5342
```

```
#> OTU2          13972          7378          4774           390          5229
#> OTU5          12167         10219          5241           910            65
#> OTU6           2473          8074          3297           668           110
#>      P01.F12.HLM140 P01.G03.HLM067 P01.G05.HLM083 P01.G06.HLM075 P01.H01.HLM063
#> OTU3              0              1              1              0              0
#> OTU4              0              0              0              0              0
#> OTU1           9264           3191           2189          15747           1467
#> OTU2           9750           2811           2108          16064           1530
#> OTU5           2061           3171           2343           1959           6545
#> OTU6           1525            964           5070           4084           5590
#>      P01.H04.HLM110 P01.H08.HLM052 P01.H11.HLM124 P01.H12.HLM148 P02.A01.HLM146
#> OTU3              0              0              1              0              0
#> OTU4              0              0              0              0              0
#> OTU1           4345           3938           7811           5929           7118
#> OTU2           4122           3937           7212           5206           6512
#> OTU5           1965           3445           4747            232           1418
#> OTU6           1500           3095           1415            582            702
#>      P02.A05.HLM167 P02.A09.HLM134 P02.A10.HLM405 P02.A11.HLM413 P02.A12.HLM421
#> OTU3              0              0          25330          18023          20357
#> OTU4              1              0          22786          16339          18961
#> OTU1          10108           1563            164          13534            376
#> OTU2           9531           1430            176          13427            389
#> OTU5            981           3447             38              1              0
#> OTU6           5955            553            146             34             85
#>      P02.B01.HLM145 P02.B04.HLM001 P02.B08.HLM003 P02.B10.HLM406 P02.B11.HLM414
#> OTU3              0              0              0           3394           1902
#> OTU4              0              0              0           3109           1869
#> OTU1            266          10678          25768           6874           5550
#> OTU2            239           9855          25621           6450           5708
#> OTU5            110            903           4434              0              0
#> OTU6             82           1617           2407             58             30
#>      P02.B12.HLM422 P02.C10.HLM407 P02.C11.HLM415 P02.C12.HLM423 P02.D09.HLM400
#> OTU3            638           7896           1663          27594          24451
#> OTU4            621           7246           1641          25516          22516
#> OTU1            324            949           2802              0              2
#> OTU2            361            900           2822              0              0
#> OTU5              0              0            369              0              0
#> OTU6             83             41             41              1              0
#>      P02.D10.HLM408 P02.D11.HLM416 P02.D12.HLM424 P02.E09.HLM401 P02.E10.HLM409
#> OTU3          13749          10195          49941          35473           3404
#> OTU4          13044           9262          46388          32911           3296
#> OTU1            490             28              9              0             76
#> OTU2            443             20              3              2             20
#> OTU5              0             20              0              0              0
#> OTU6            101             28              1              0             14
#>      P02.E11.HLM417 P02.E12.HLM425 P02.F09.HLM402 P02.F10.HLM410 P02.F11.HLM418
#> OTU3          14098            861           7180            569           5554
#> OTU4          13335            801           6663            622           5022
#> OTU1              2              1            169            156           1462
#> OTU2              1              0            169            123           1565
#> OTU5              1              1              0              0            146
#> OTU6              0              3             57             48             46
#>      P02.F12.HLM426 P02.G03.HLM036 P02.G09.HLM403 P02.G10.HLM411 P02.G11.HLM419
```

```
#> OTU3           3093              0          37534            477          10683
#> OTU4           2846              0          34974            488          10204
#> OTU1            303          12443              0             78           3551
#> OTU2            301          11548              1            100           3401
#> OTU5             97            136              0              1              0
#> OTU6              4            232              0            259            158
#>      P02.G12.HLM427 P02.H02.HLM176 P02.H04.HLM162 P02.H06.HLM152 P02.H09.HLM404
#> OTU3           4538              0              0              1           9189
#> OTU4           4331              0              0              1           8597
#> OTU1            456          12289           6043           9164              1
#> OTU2            454          11797           5930           8558              0
#> OTU5             23            555           4398           2433              0
#> OTU6              6            591           2052            870              0
#>      P02.H10.HLM412 P02.H11.HLM420 P02.H12.HLM428 P03.A01.HLM429 P03.B01.HLM430
#> OTU3          22068           8178           6681           3386          17435
#> OTU4          20503           7712           6297           3218          16101
#> OTU1            131             13              4            237           1147
#> OTU2            180            145              1            260           1172
#> OTU5              0              0              0              0              0
#> OTU6             69            410             12              6              8
#>      P03.C01.HLM431 P03.D01.NEC
#> OTU3             47           0
#> OTU4             36           0
#> OTU1             31           0
#> OTU2             21           0
#> OTU5              1           0
#> OTU6            158           0

# Inspect the first few rows of the taxonomy table
head(tax_df)
#>         Kingdom     Phylum   Class              Order                    Family
#> OTU120 Bacteria Firmicutes Bacilli Erysipelotrichales       Erysipelotrichaceae
#> OTU524 Bacteria Firmicutes Bacilli Erysipelotrichales       Erysipelotrichaceae
#> OTU598 Bacteria Firmicutes Bacilli Erysipelotrichales       Erysipelotrichaceae
#> OTU662 Bacteria Firmicutes Bacilli Erysipelotrichales Erysipelatoclostridiaceae
#> OTU725 Bacteria Firmicutes Bacilli Erysipelotrichales Erysipelatoclostridiaceae
#> OTU460 Bacteria Firmicutes Bacilli  Acholeplasmatales        Acholeplasmataceae
#>                              Genus                    Species
#> OTU120                     Unknown                    Unknown
#> OTU524                   Bulleidia         Bulleidia extructa
#> OTU598 Erysipelotrichaceae UCG-006                    Unknown
#> OTU662                     Unknown                    Unknown
#> OTU725                   Eggerthia     Eggerthia catenaformis
#> OTU460                Acholeplasma Mycoplasmataceae genomosp.

# Inspect the first few rows of Genexpert metadata
head(Genexpert_metadata_df)
#>                    Group
#> P01.A01.HLM069 Xpert-ve
#> P01.A02.HLM002 Xpert-ve
#> P01.A03.HLM038 Xpert-ve
#> P01.A04.HLM091 Xpert-ve
#> P01.A05.HLM061 Xpert-ve
```

```
#> P01.A06.HLM094 Xpert-ve

# Inspect the first few rows of Mtb Infection Status metadata
head(MtbInfectionStatus_metadata_df)
#>                          Group
#> P01.A06.HLM094 M.tb_Uninfected
#> P01.A07.HLM102            LTBI
#> P01.A09.HLM053            LTBI
#> P01.A11.HLM137 M.tb_Uninfected
#> P01.A12.HLM131 M.tb_Uninfected
#> P01.B10.HLM020 M.tb_Uninfected
```

Convert each OTU, Taxonomy and Metadata file into a matrix

```
# Create the OTU table for Genexpert samples
Genexpert_OTU <- otu_table(Genexpert_otu_df, taxa_are_rows = TRUE)

# Create the OTU table for Mtb Infection Status samples
MtbInfectionStatus_OTU <- otu_table(MtbInfectionStatus_otu_df, taxa_are_rows = TRUE)

# Create the taxonomy table
TAX <- phyloseq::tax_table(as.matrix(tax_df))

# Create the sample data for Genexpert samples
Genexpert_SAM <- sample_data(Genexpert_metadata_df)

# Create the sample data for Mtb Infection Status samples
MtbInfectionStatus_SAM <- sample_data(MtbInfectionStatus_metadata_df)
```

Create phyloseq object

This is an S4 object required for downstream analysis.

```
# Create the phyloseq object for Genexpert samples
Genexpert_phyloseq <- phyloseq(Genexpert_OTU, TAX, Genexpert_SAM)

# Create the phyloseq object for Mtb Infection Status samples
MtbInfectionStatus_phyloseq <- phyloseq(MtbInfectionStatus_OTU, TAX, MtbInfectionStatus_SAM)
```

Inspect the phyloseq object

```
# Inspect the Genexpert phyloseq object
Genexpert_phyloseq
#> phyloseq-class experiment-level object
#> otu_table()   OTU Table:         [ 835 taxa and 193 samples ]
#> sample_data() Sample Data:       [ 193 samples by 1 sample variables ]
#> tax_table()   Taxonomy Table:    [ 835 taxa by 7 taxonomic ranks ]
```

```
# Inspect the Mtb Infection Status phyloseq object
MtbInfectionStatus_phyloseq
#> phyloseq-class experiment-level object
#> otu_table()   OTU Table:         [ 500 taxa and 72 samples ]
#> sample_data() Sample Data:       [ 72 samples by 1 sample variables ]
#> tax_table()   Taxonomy Table:    [ 500 taxa by 7 taxonomic ranks ]
```

Save phyloseq objects

```
# Save Genexpert_physeq as an RDS file
saveRDS(Genexpert_phyloseq, file = "data/Genexpert_physeq.rds")

# Save MtbInfectionStatus_physeq as an RDS file
saveRDS(MtbInfectionStatus_phyloseq, file = "data/MtbInfectionStatus_physeq.rds")
```

Diversity metrices

Alpha diversity

```
metrics <- c("Shannon", "Observed", "Chao1", "Simpson", "Fisher", "ACE")
pal.Coll <- c("darkblue", "coral", "darkgreen")

# Estimate richness for Genexpert data
alpha_metrics_Group <- estimate_richness(Genexpert_phyloseq, measures = metrics)

# Estimate richness for MtbStatus data
alpha_metrics_MtbStatus <- estimate_richness(MtbInfectionStatus_phyloseq, measures = metrics)

# Combine Genexpert metadata with richness estimates and adjust the Group variable
alpha_metrics_df_Group <- cbind(Genexpert_metadata_df, alpha_metrics_Group)
alpha_metrics_df_Group$Group <- as.factor(alpha_metrics_df_Group$Group)

# Combine MtbStatus metadata with richness estimates and adjust the Group variable
alpha_metrics_df_MtbStatus <- cbind(MtbInfectionStatus_metadata_df, alpha_metrics_MtbStatus)
alpha_metrics_df_MtbStatus$Group <- as.factor(alpha_metrics_df_MtbStatus$Group)
```

Function for generating Mtb Infection status plots

```
# Function to generate a plot for a given metric
generate_plot <- function(metric) {
  # Perform Wilcoxon test on the metric grouped by 'Group', adjust p-values using BH method,
  # add significance and position information
  stats_test <- alpha_metrics_df_MtbStatus %>%
    rstatix::wilcox_test(reformulate("Group", metric)) %>%
    rstatix::adjust_pvalue(method = "BH") %>%
    rstatix::add_significance() %>%
```

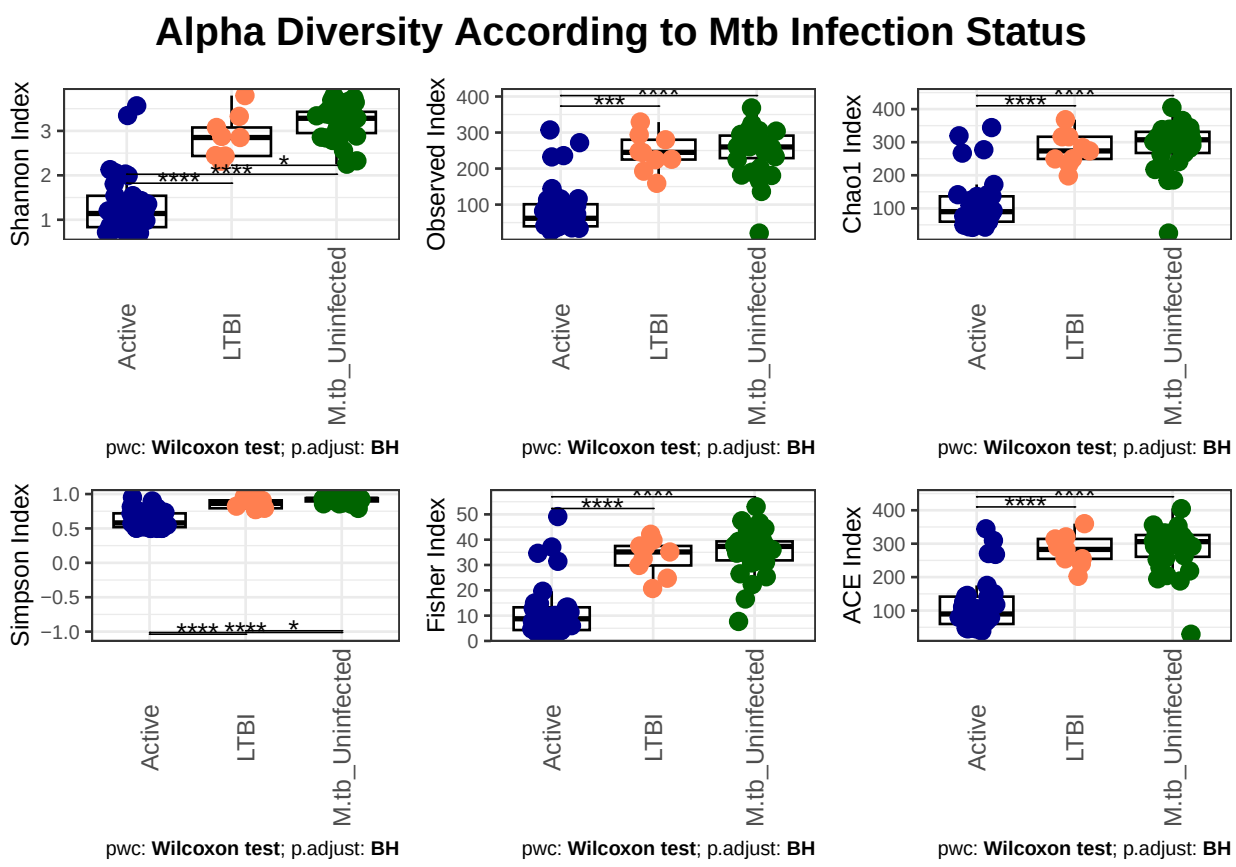
```
    rstatix::add_xy_position(x = "Group")

  # Create the plot
  plot <- alpha_metrics_df_MtbStatus %>%
    ggplot(aes(x = Group, y = .data[[metric]])) +
    geom_boxplot(color = "black", alpha = 0.5, outlier.shape = NA) +  # Add boxplot
    geom_point(position = position_jitter(0.2), size = 3, aes(fill = Group, color = Group)) +  # Add po
    scale_color_manual(values = pal.Coll) +  # Set manual color scale
    xlab("Group") +  # Label x-axis
    ylab(paste(metric, "Index")) +  # Label y-axis
    labs(caption = get_pwc_label(stats_test)) +  # Add caption with significance
    theme_bw() +  # Set theme to black and white
    theme(
      text = element_text(size = 10),  # Set text size
      axis.title.x = element_blank(),  # Remove x-axis title
      axis.text.x = element_blank(),  # Remove x-axis text
      axis.ticks.x = element_blank(),  # Remove x-axis ticks
      axis.text.x.bottom = element_text(size = 10, angle = 90),  # Set x-axis text at bottom with rotat
      legend.position = "none"  # Remove legend
    ) +
    stat_pvalue_manual(stats_test, bracket.nudge.y = -2, step.increase = 0.05, hide.ns = TRUE, tip.leng

  # Return the plot and statistical test results as a list
  return(list(plot = plot, stats_test = stats_test))
}
```

Generate cow plot

```
plots <- lapply(metrics, generate_plot)
names(plots) <- metrics
alpha_diversity_final <- cowplot::plot_grid(plotlist = lapply(plots, `[[`, "plot"))
# Add a title to the alpha_diversity_final plot
alpha_diversity_final <- ggdraw() +
  draw_plot(alpha_diversity_final, 0, 0, 1, 0.9) +  # Adjust plot size to leave space for title
  draw_label("Alpha Diversity According to Mtb Infection Status",
             x = 0.5, y = 0.95, hjust = 0.5, size = 16, fontface = "bold")
```

View cow plot

```
print(alpha_diversity_final)
```




```
ggsave(filename = "Results/Figure 1.jpg",
       plot = alpha_diversity_final,
       width = 10, height = 8, dpi = 600)
```

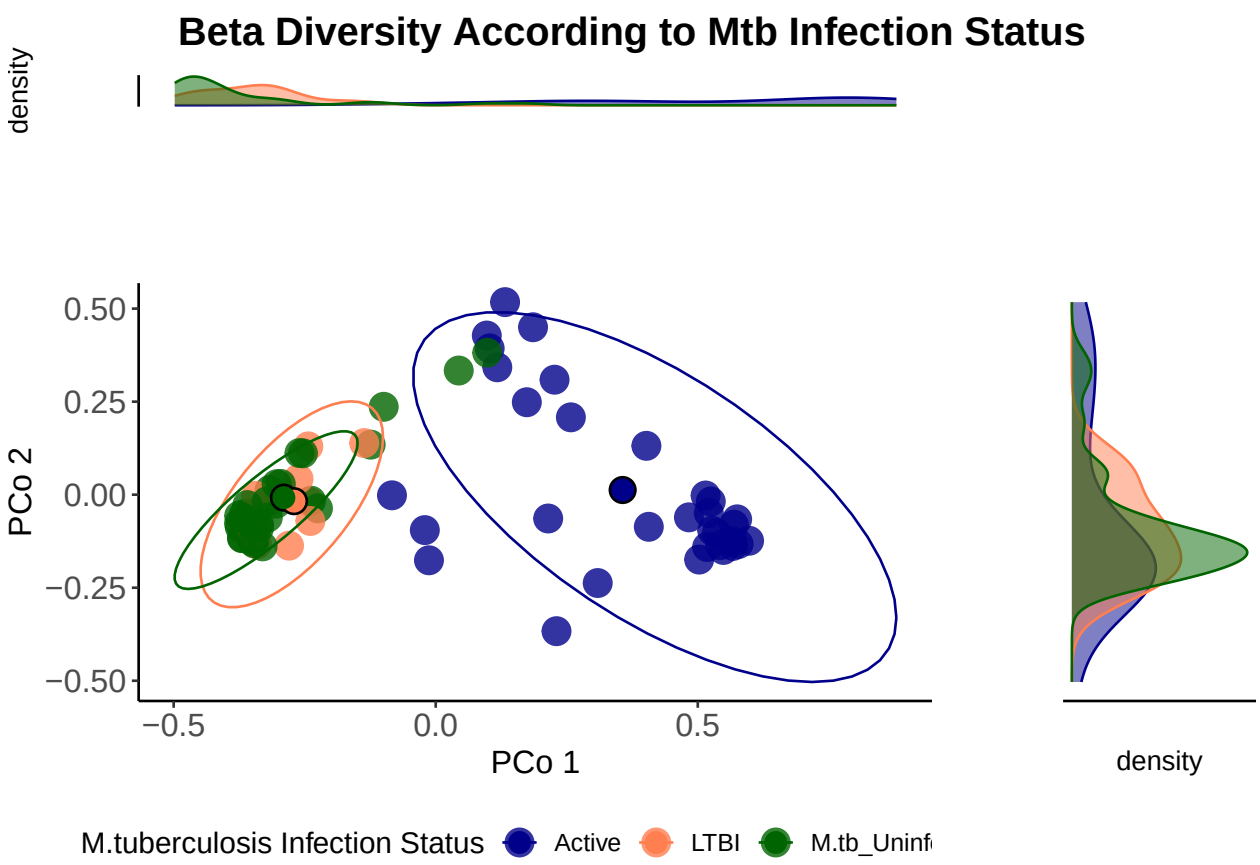
Beta diversity

This is done using the Bray_Curtis dissimilarity matrix.

```
# Compute Bray-Curtis dissimilarity matrix and perform PCoA for Genexpert results
beta <- vegan::vegdist(t(Genexpert_otu_df), method = "bray", na.rm = TRUE)
beta[is.na(beta)] <- 0
pcoaE <- cmdscale(beta, k = 2)
pcoaE <- as.data.frame(pcoaE)
metadata_beta <- cbind(Genexpert_metadata_df, pcoaE)
Group_metadata_beta <- metadata_beta
centroids <- aggregate(cbind(V1, V2) ~ Group, metadata_beta, mean)
# Compute Bray-Curtis dissimilarity matrix and perform PCoA for Mtb Infection status
beta <- vegan::vegdist(t(MtbInfectionStatus_otu_df), method = "bray", na.rm = TRUE)  # Compute Bray-Cur
beta[is.na(beta)] <- 0  # Replace any NA values with 0
pcoaE <- cmdscale(beta, k = 2)  # Perform PCoA, reducing to 2 dimensions
pcoaE <- as.data.frame(pcoaE)  # Convert PCoA result to a data frame
metadata_beta <- cbind(MtbInfectionStatus_metadata_df, pcoaE)  # Combine metadata with PCoA results
centroids <- aggregate(cbind(V1, V2) ~ Group, metadata_beta, mean)  # Calculate centroids for each grou
```

```r
# Plot PCoA with points colored by Group
metadata_beta %>%
  ggplot(aes(x = V1, y = V2, color = Group)) +  # PCoA plot by M.tb infection status
  theme_classic() +
  scale_color_manual(values = pal.Coll) +  # Set custom colors for groups
  geom_point(aes(color = Group), size = 5, alpha = 0.8) +  # Plot points with transparency
  xlab("PCo 1") + ylab("PCo 2") +  # Label axes
  theme(axis.title.x = element_text(size = 13),
        axis.text.x = element_text(size = 13),
        axis.text.y = element_text(size = 13),
        axis.title.y = element_text(size = 13),
        legend.position = "bottom",  # Position the legend on the bottom
        legend.title = element_text(size = 12),  # Set title size for legend
        legend.text = element_text(size = 10)) +  # Set text size for legend
  labs(color = "M.tuberculosis Infection Status") +  # Set legend title
  stat_ellipse(aes(color = Group)) +  # Add ellipses to show group clusters
  geom_point(data = centroids, size = 5, shape = 16, color = "black") + # Add centroids in black
  geom_point(data = centroids, size = 4, shape = 16) -> B  # Overlay centroids with group color

# Add density plot for V1 (first PCoA axis)
xdensity <- metadata_beta %>%
  ggplot(aes(x = V1)) +
  geom_density(alpha = 0.5, aes(fill = Group, color = Group)) +  # Create density plot
  scale_fill_manual(values = pal.Coll) +  # Set manual fill colors
  scale_color_manual(values = pal.Coll) +  # Set manual line colors
  theme_classic() +  # Apply classic theme for a clean look
  theme(
    axis.title.x = element_blank(),  # Remove x-axis title
    axis.text.x = element_blank(),  # Remove x-axis text
    axis.ticks.x = element_blank(),  # Remove x-axis ticks
    axis.line.x = element_blank(),  # Remove x-axis line
    axis.text.y = element_blank(),  # Remove y-axis text
    axis.ticks.y = element_blank(),  # Remove y-axis ticks
    legend.position = "none"  # Hide legend
  )

# Add density plot for V2 (second PCoA axis)
ydensity <- metadata_beta %>%
  ggplot(aes(V2)) +
  geom_density(alpha = 0.5, aes(fill = Group, color = Group)) +  # Create density plot for V2 colored b
  scale_fill_manual(values = pal.Coll) +  # Set manual fill colors
  scale_color_manual(values = pal.Coll) +  # Set manual line colors
  theme_classic() +  # Apply classic theme for a clean look
  theme(
    axis.text.x = element_blank(),  # Remove x-axis text
    axis.ticks.x = element_blank(),  # Remove x-axis ticks
    axis.title.y = element_blank(),  # Remove y-axis title
    axis.text.y = element_blank(),  # Remove y-axis text
    axis.ticks.y = element_blank(),  # Remove y-axis ticks
    axis.line.y = element_blank(),  # Remove y-axis line
    legend.position = "none"  # Hide legend
  ) +
  coord_flip()  # Flip coordinates for a vertical plot
```

```r
# Create a blank plot for spacing
blankPlot <- ggplot() + geom_blank(aes(1,1)) + theme_void()

# Create a combined plot with legend at the bottom
beta_final <- plot_grid(
  xdensity + theme(plot.margin = unit(c(0, 0, 0, 0), "cm")),  # Add x-density plot with no margins
  blankPlot + theme(plot.margin = unit(c(0, 0, 0, 0), "cm")),  # Add a blank plot with no margins
  B + theme(legend.position = "bottom",  # Add the main plot B with legend at the bottom
            plot.margin = unit(c(0, 0, 0, 0), "cm")),
  ydensity + theme(plot.margin = unit(c(0, 0, 0, 0), "cm")),  # Add y-density plot with no margins
  nrow = 2,  # Arrange plots in a 2-row grid
  rel_widths = c(4, 1.4),  # Set relative widths for columns
  rel_heights = c(1.4, 4),  # Set relative heights for rows
  align = "hv"  # Align plots both horizontally and vertically
)
# Load cowplot package
library(cowplot)

# Add a title to the beta_final plot
beta_final <- ggdraw() +
  draw_plot(beta_final, 0, 0, 1, 0.9) +  # Adjust the plot size to leave space for the title
  draw_label("Beta Diversity According to Mtb Infection Status",
             x = 0.5, y = 0.95, hjust = 0.5, size = 16, fontface = "bold")
# Display the final plot
print(beta_final)
```




```
# Perform PERMANOVA test to compare beta diversity between 'TB_history' groups
# Compute Bray-Curtis dissimilarity matrix from OTU data
permanova <- adonis2(vegdist(t(MtbInfectionStatus_otu_df), method = "bray", na.rm = TRUE) ~ as.factor(G

# Convert PERMANOVA results to a data frame
permanova <- as.data.frame(permanova)

# Save the data frame to a CSV file
write.csv(permanova, file = "Results/Mtb_infection status_permanova_results.csv", row.names = TRUE)

ggsave(filename = "Results/Figure 2.jpg",
       plot = beta_final,
       width = 10, height = 8, dpi = 600)
```

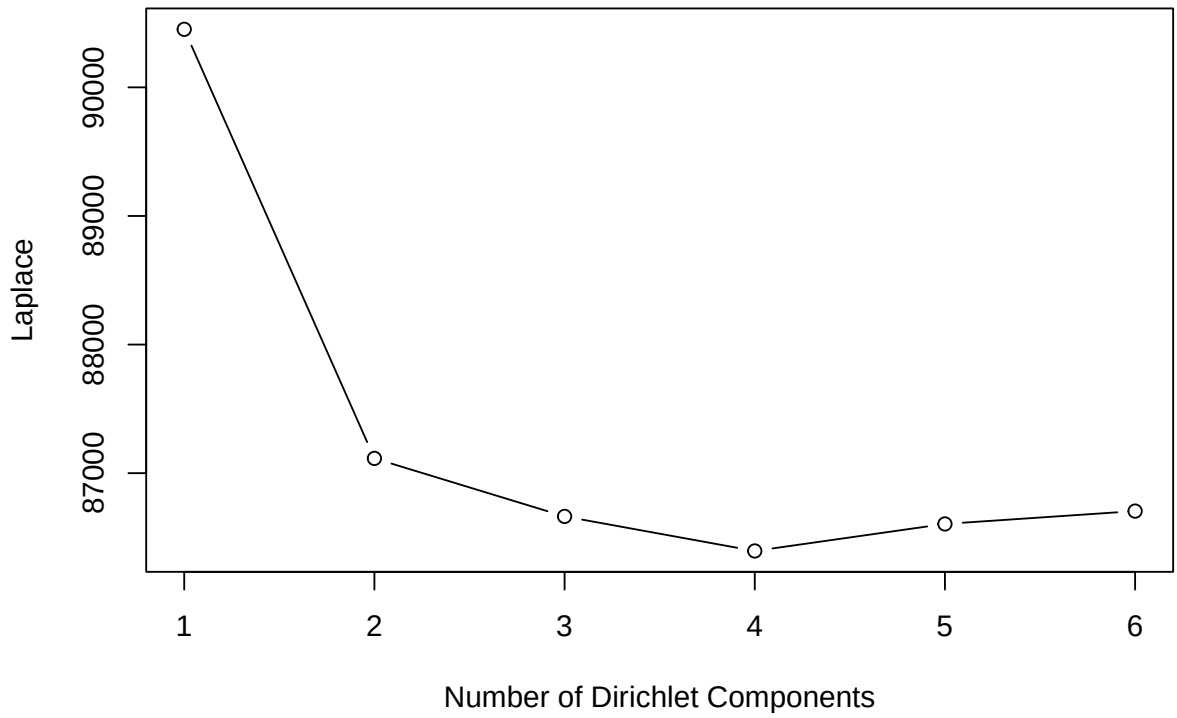
DMM

The models help identify underlying community structures by partitioning the data into distinct groups based on microbial abundance profiles. DMM utilizes an Infinite mixture model; hence, it can infer the optimal number of microbiome microbial community types.

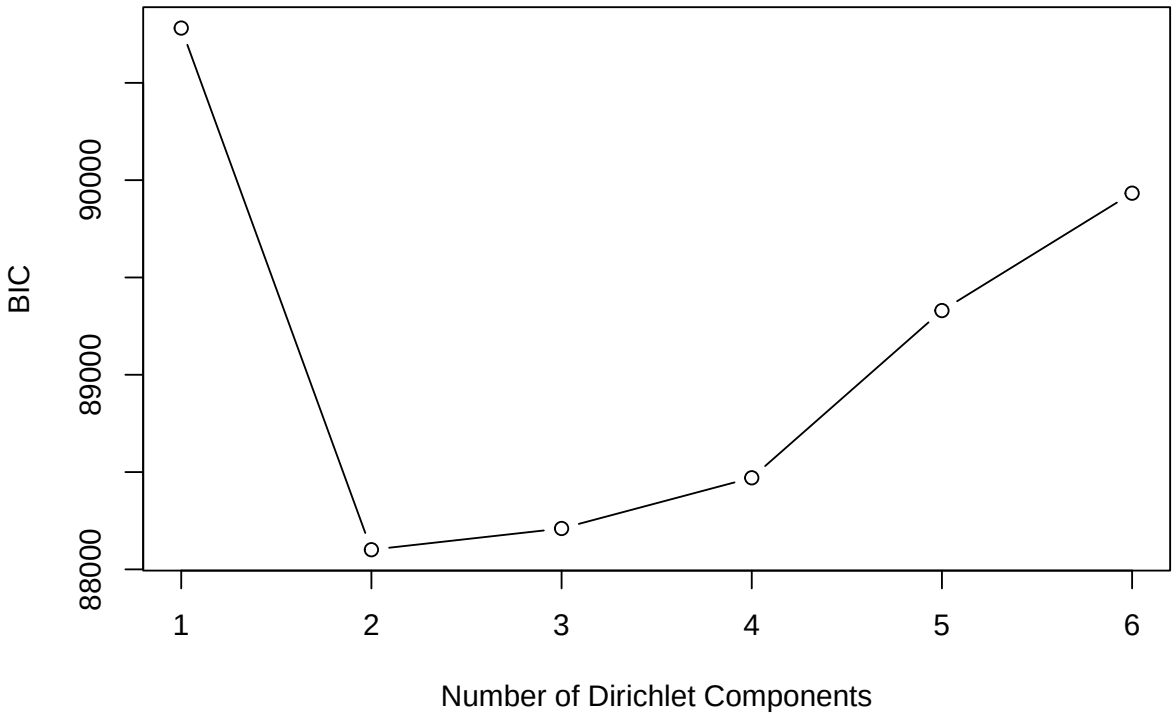
```
# Read in the Genus abundance table
genus <- read.table("data/Genus.txt", header = TRUE, row.names = NULL, sep = "\t")
```

```r
# Assign tax column as row names and remove the tax column from the data frame
rownames(genus) <- genus$Genus
genus <- subset(genus, select = -Genus)

# Ensure row names are unique
unique_row_names <- make.names(row.names(genus), unique = TRUE)
row.names(genus) <- unique_row_names

# Create a copy of genus called raw
raw <- genus

# Transpose the data frame and remove columns with zero sums
raw_values <- t(raw)  # Transpose the data frame
raw_values <- raw_values[, colSums(raw_values) > 0]  # Remove columns with zero sums
raw_values_t <- t(raw_values)  # Transpose back to the original orientation

# Note that genus_table has samples as columns and genera as rows
genus_table <- as.data.frame(raw_values_t)

# Create a data frame with the row names of genus_table
genus_p <- as.data.frame(row.names(genus_table))

# Rename the column to 'long_names'
colnames(genus_p) <- "long_names"

# Separate the long names into multiple columns based on the dot separator
tax_names <- tidyr::separate(
  genus_p,
  long_names,
  into = c("A", "B", "C", "D", "E", "F"),
  sep = "\\.",
  fill = "right",  # Fill missing values with NA on the right side
  extra = "drop"   # Drop any extra columns beyond what is specified
)

# Add the genus names (last part after the last dot) to genus_table
genus_table$short <- tax_names$F

# Make these genus names unique and set them as row names for genus_table
row.names(genus_table) <- make.names(genus_table$short, unique = TRUE)

# Remove the 'short' column from genus_table since it's now redundant
genus_table$short <- NULL

# Normalize genus_table
genus_table <- genus_table / min(genus_table[genus_table > 0])

# Set the maximum number of Dirichlet components to check
all_dmns <- 6

# Initialize a list to store the Dirichlet models
dmn_list <- vector("list", all_dmns)
```

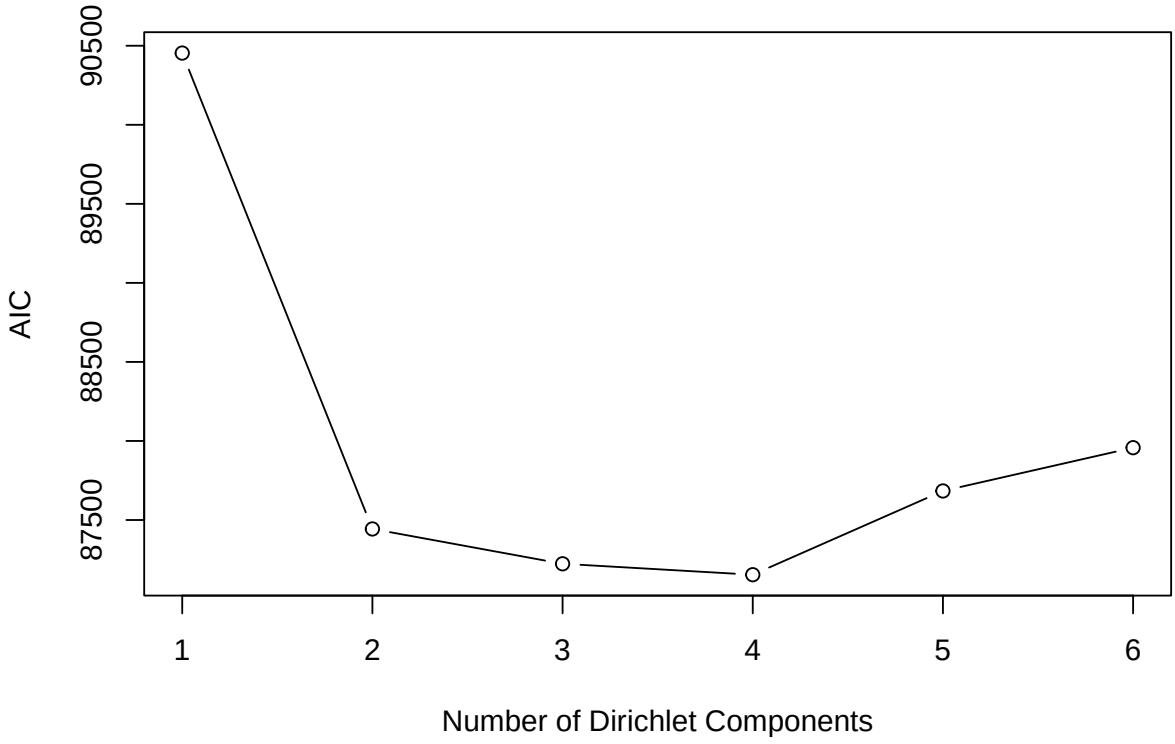
```r
# Fit Dirichlet Multinomial models with components ranging from 1 to all_dmns
for (i in 1:all_dmns) {
  print(i)  # Print the current number of components being processed
  dmn_list[[i]] <- dmn(as.matrix(t(genus_table)), i, verbose = FALSE)  # Fit the model and store it in
}
#> [1] 1
#> [1] 2
#> [1] 3
#> [1] 4
#> [1] 5
#> [1] 6

# Calculate Laplace, BIC, and AIC for each Dirichlet Multinomial model
lplc <- sapply(dmn_list, laplace)
BIC <- sapply(dmn_list, BIC)
AIC <- sapply(dmn_list, AIC)

# Plot Laplace, BIC, and AIC values
plot(lplc, type = "b", xlab = "Number of Dirichlet Components", ylab = "Laplace")
```




```r
plot(BIC, type = "b", xlab = "Number of Dirichlet Components", ylab = "BIC")
```




```
plot(AIC, type = "b", xlab = "Number of Dirichlet Components", ylab = "AIC")
```




```
# Find the model with the minimum Laplace value
best_lplc_index <- which.min(lplc)
optimal_lplc_model <- dmn_list[[best_lplc_index]]
print(optimal_lplc_model)
#> class: DMN
#> k: 4
#> samples x taxa: 193 x 201
#> Laplace: 86394.96 BIC: 88470.49 AIC: 87153.99

# Find the model with the minimum BIC value
best_BIC_index <- which.min(BIC)
optimal_BIC_model <- dmn_list[[best_BIC_index]]
print(optimal_BIC_model)
#> class: DMN
#> k: 2
#> samples x taxa: 193 x 201
#> Laplace: 87114.73 BIC: 88100.98 AIC: 87443.55

# Find the model with the minimum AIC value
best_AIC_index <- which.min(AIC)
optimal_AIC_model <- dmn_list[[best_AIC_index]]
print(optimal_AIC_model)
#> class: DMN
#> k: 4
```

```
#> samples x taxa: 193 x 201
#> Laplace: 86394.96 BIC: 88470.49 AIC: 87153.99

# Assign the best model based on the minimum Laplace value as the final model
best_fit <- dmn_list[[best_lplc_index]]

# Get the cluster importance from the best model
cluster_imp <- fitted(best_fit)  # Extract cluster importance

# Print the cluster importance
print(cluster_imp)
#>                                 [,1]         [,2]         [,3]         [,4]
#> X                       1.263659e-02 2.362066e-02 0.0202747402 2.304541e-02
#> X.1                     2.683149e-01 4.244615e-01 0.0493113768 2.834029e-01
#> X.2                     2.185983e-03 7.279051e-03 0.0003919807 8.100703e-03
#> Actinomyces             7.819487e-01 1.642459e+00 0.1149420428 8.573350e-01
#> F0332                   4.077522e-02 1.048952e-01 0.0043109362 8.493943e-02
#> Mobiluncus              1.009968e-02 4.169227e-02 0.0043250332 5.523952e-02
#> Alloscardovia           2.270671e-02 3.167668e-02 0.0122506716 3.867948e-02
#> Bifidobacterium         2.946250e-02 6.031145e-02 0.0206211374 6.287382e-02
#> Parascardovia           6.155104e-03 2.151436e-02 0.0003919807 8.213079e-03
#> X.3                     1.425040e-02 1.687462e-02 0.0042941805 3.124205e-02
#> Corynebacterium         1.953718e-01 3.011097e-01 0.0492037199 1.806635e-01
#> Mycobacterium           1.955758e-02 2.152256e-02 1.8367784992 2.238605e-02
#> Tropheryma              6.154352e-03 1.470503e-02 0.0003919806 3.167916e-02
#> Microbacterium          4.029153e-02 8.573785e-02 0.0941273714 9.761745e-02
#> X.4                     2.019823e-01 2.762347e-01 0.0620470414 1.584808e-01
#> Rothia                  7.130977e-01 1.726093e+00 0.1098843750 5.413843e-01
#> X.5                     4.052812e-02 8.397873e-02 0.0082782768 8.058769e-02
#> Brooklawnia             2.049684e-04 1.209833e-02 0.0042941805 8.074897e-03
#> Cutibacterium           2.222797e-02 2.389073e-02 0.1087830809 7.224155e-04
#> Propionibacterium       4.092445e-02 1.056958e-01 0.0121554847 7.462606e-02
#> Pseudopropionibacterium 1.101226e-01 2.462330e-01 0.0167635610 3.339463e-01
#> Streptomyces            2.991793e-02 2.567371e-02 0.0695800084 3.708818e-02
#> Sphaerimonospora        2.016848e-04 2.338378e-04 0.0303051443 7.224209e-04
#> Atopobium               2.995692e-01 6.206299e-01 0.0816583500 6.818355e-01
#> Olsenella               3.284866e-02 1.253960e-01 0.0204416318 1.549501e-01
#> Cryptobacterium         8.217301e-03 4.903217e-02 0.0082345207 8.763964e-02
#> DNF00809                1.015096e-02 1.910726e-02 0.0003919807 3.880776e-02
#> Slackia                 2.448725e-02 7.951178e-02 0.0202225903 1.077304e-01
#> X.6                     1.015826e-02 3.116151e-02 0.0003919807 7.942535e-03
#> X.7                     2.185975e-03 7.330385e-03 0.0003919807 7.942454e-03
#> X.8                     1.229179e-02 2.435877e-02 0.0003919807 2.310727e-02
#> Bacteroides             1.229050e-02 3.765894e-02 0.0003919807 3.813679e-02
#> Incertae                1.422210e-02 5.881976e-02 0.0003919807 4.637299e-02
#> X.9                     2.173308e-03 3.224319e-02 0.0003919807 1.527257e-02
#> uncultured              4.170693e-03 3.460819e-02 0.0003919807 2.326689e-02
#> Odoribacter             2.186067e-03 3.265997e-02 0.0003919807 1.527264e-02
#> F0058                   6.653482e-02 1.359224e-01 0.0081980873 9.647801e-02
#> X.10                    1.616147e-02 1.684573e-02 0.0003919806 7.224256e-04
#> Porphyromonas           6.184014e-01 2.322812e+00 0.0240436252 4.780314e-01
#> X.11                    5.064878e-01 9.291493e-01 0.0082460411 3.051290e-01
#> Alloprevotella          9.304271e-01 2.006020e+00 0.0204821249 5.260670e-01
```

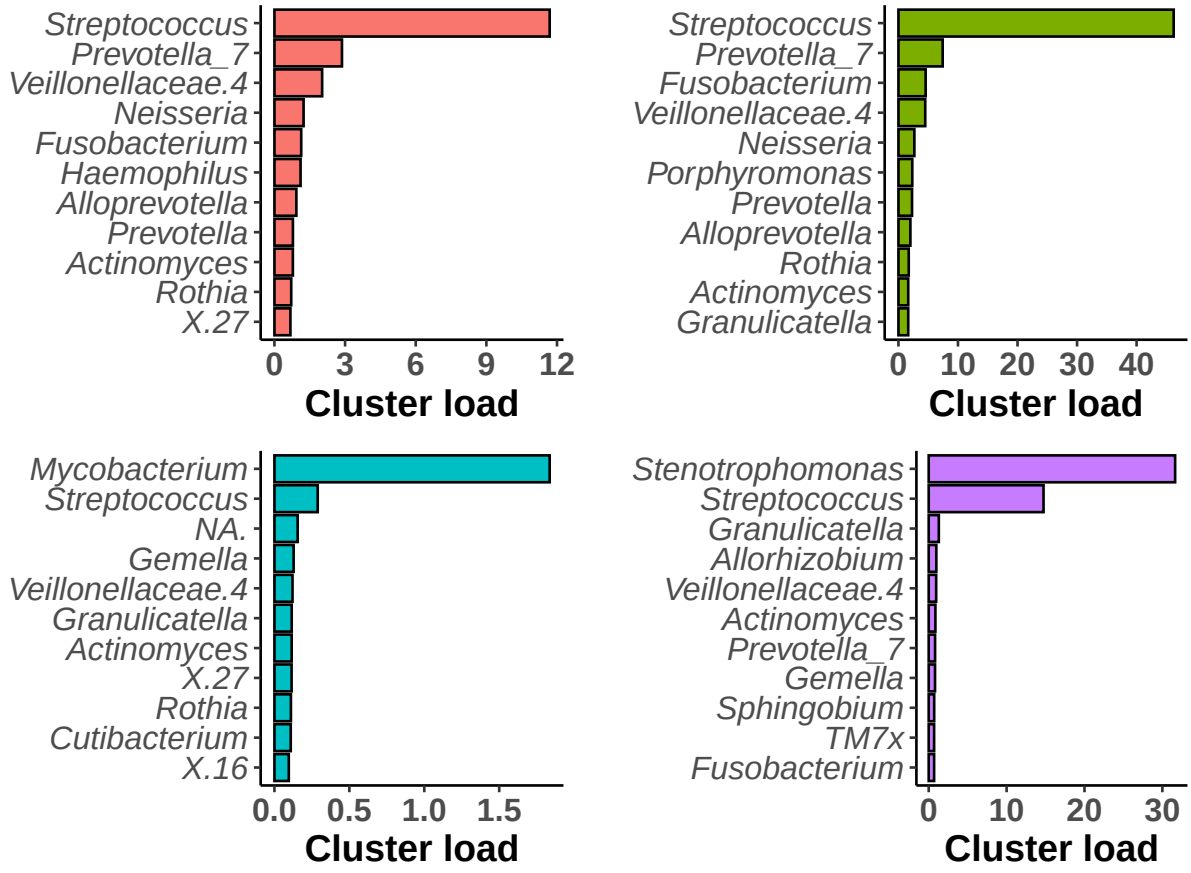
```
#> Prevotella              7.843064e-01 2.279899e+00 0.0287079976 4.787595e-01
#> Prevotella_7            2.867297e+00 7.428274e+00 0.0506945489 8.158666e-01
#> Prevotellaceae          4.222777e-03 2.626761e-02 0.0003919807 7.999757e-03
#> Rikenellaceae           1.021712e-02 7.443530e-02 0.0003919806 6.137026e-02
#> Tannerella              1.728072e-01 3.048391e-01 0.0042941805 1.629837e-01
#> X2534                   6.135874e-03 1.698494e-02 0.0003919807 8.123737e-03
#> Edaphobaculum           4.200921e-03 3.209695e-02 0.0043186667 4.222548e-02
#> Capnocytophaga          2.425102e-01 3.920230e-01 0.0043420156 2.610369e-01
#> Bergeyella              1.392331e-01 1.883846e-01 0.0003919806 1.114454e-01
#> X.12                    4.200403e-03 1.994186e-02 0.0003919806 7.999757e-03
#> Lentimicrobium          5.241897e-02 1.093879e-01 0.0003919807 7.269406e-02
#> X.13                    4.802178e-02 1.549031e-01 0.0003919806 5.512951e-02
#> Campylobacter           2.772387e-01 4.676444e-01 0.0161807345 2.502364e-01
#> Helicobacter            2.208859e-03 2.582973e-03 0.0003919807 7.224259e-04
#> Wolinella               6.137332e-03 2.180308e-02 0.0003919807 7.224258e-04
#> Flexilinea              2.152791e-04 1.444123e-02 0.0003919807 3.734995e-02
#> X.14                    2.870004e-02 5.265428e-02 0.0286497432 4.793264e-02
#> aestivum                1.431413e-02 1.208552e-02 0.0081980873 2.324184e-02
#> Desulfomicrobium        2.196656e-03 1.926281e-02 0.0003919807 7.999757e-03
#> Acholeplasma            6.153357e-03 2.539876e-02 0.0003919807 7.224258e-04
#> Brevibacillus           4.194837e-03 7.313138e-03 0.0670572194 7.221838e-04
#> X.15                    6.244360e-03 3.967611e-02 0.0003919807 7.224258e-04
#> Eggerthia               2.185976e-03 4.128300e-02 0.0003919807 3.748462e-02
#> X.16                    1.946983e-01 3.476894e-01 0.0956640142 4.628765e-01
#> Bulleidia               1.018909e-02 6.223600e-02 0.0043109362 8.151460e-02
#> Erysipelotrichaceae     8.180739e-03 4.833624e-02 0.0003919807 5.596066e-02
#> X.17                    1.387484e-01 2.395767e-01 0.0285331388 1.844661e-01
#> Abiotrophia             1.708754e-01 2.975688e-01 0.0502565134 3.260757e-01
#> Dolosigranulum          1.057915e-02 2.203338e-02 0.0042941805 8.128351e-03
#> Granulicatella          6.395926e-01 1.641031e+00 0.1151874196 1.296226e+00
#> X.18                    2.275930e-02 5.882444e-02 0.0043109362 3.865201e-02
#> Lacticaseibacillus      2.016848e-04 2.582973e-03 0.0003919807 8.244204e-03
#> Lactobacillus           1.239644e-02 3.274888e-02 0.0042941805 1.566798e-02
#> Ligilactobacillus       3.222627e-02 4.905563e-02 0.0003919807 2.350252e-02
#> Limosilactobacillus     4.142252e-02 9.988487e-02 0.0043420156 7.158277e-02
#> Weissella               1.429624e-02 1.456510e-02 0.0043109362 2.275589e-02
#> X.19                    1.485492e-01 2.316665e-01 0.0199164412 1.900658e-01
#> Streptococcus           1.168768e+01 4.622996e+01 0.2888309317 1.474181e+01
#> Mycoplasma              2.765916e-02 1.255529e-01 0.0003919807 6.396092e-02
#> Paenibacillus           2.016848e-04 2.338348e-04 0.0085270011 7.224204e-04
#> X.20                    3.544600e-02 6.095152e-02 0.0003919806 6.183006e-02
#> oral                    2.235912e-02 6.182924e-02 0.0003919806 3.869413e-02
#> X.21                    1.383631e-01 1.940173e-01 0.0338619577 1.543555e-01
#> Gemella                 5.810720e-01 1.364337e+00 0.1280248351 8.101043e-01
#> Staphylococcus          3.341576e-02 4.692248e-02 0.0741199465 8.881651e-02
#> X014                    1.811266e-01 3.602400e-01 0.0209090021 2.800263e-01
#> X014.1                  1.013540e-01 2.219069e-01 0.0164013456 2.273919e-01
#> group                   2.016848e-04 1.219598e-02 0.0003919807 8.100703e-03
#> group.1                 1.868075e-02 8.388716e-02 0.0042941905 1.547656e-02
#> Pseudoramibacter        8.127772e-03 3.821936e-02 0.0043271228 6.046749e-02
#> Defluviitaleaceae       1.630978e-02 1.153693e-01 0.0003919806 4.760644e-02
#> X.22                    2.719237e-01 4.870799e-01 0.0672744910 3.539753e-01
#> Butyrivibrio            1.098836e-01 1.984701e-01 0.0003919806 1.576043e-01
```

```
#> Catonella                1.517161e-01 2.831003e-01 0.0003919806 1.770038e-01
#> Howardella               1.414799e-02 3.388661e-02 0.0003919807 5.438608e-02
#> Johnsonella              9.181395e-02 2.046522e-01 0.0291021239 1.684475e-01
#> Lachnoanaerobaculum      2.081763e-01 3.524909e-01 0.0699732991 2.691597e-01
#> Moryella                 2.016848e-04 7.355410e-03 0.0003919807 7.224264e-04
#> Oribacterium             3.478836e-01 6.847996e-01 0.0779239190 5.184962e-01
#> Roseburia                8.139003e-03 4.487948e-02 0.0003919807 8.136648e-03
#> Shuttleworthia           7.239121e-02 1.892725e-01 0.0205092808 1.259943e-01
#> Stomatobaculum           2.476990e-01 4.279215e-01 0.0204401864 2.890795e-01
#> X.23                     1.019040e-02 7.349083e-03 0.0003919806 7.224258e-04
#> Eubacterium              2.185975e-03 1.920288e-02 0.0043109362 2.346864e-02
#> Peptococcus              8.207038e-02 1.486801e-01 0.0003919806 1.176224e-01
#> X.24                     1.004504e-02 7.773689e-02 0.0043109362 6.630929e-02
#> Anaerovoracaceae         8.060436e-03 8.115827e-02 0.0082568170 5.821591e-02
#> Anaerovoracaceae.1       3.555107e-02 8.555250e-02 0.0003919806 3.909252e-02
#> Anaerovoracaceae.2       2.016848e-04 4.938700e-03 0.0003919807 3.245352e-02
#> Anaerovoracaceae.3       2.425670e-02 9.671877e-02 0.0003919807 5.561243e-02
#> Anaerovoracaceae.4       9.294744e-02 1.878933e-01 0.0375878234 2.233484e-01
#> Anaerovoracaceae.5       4.433546e-02 1.242135e-01 0.0291584125 8.815872e-02
#> Anaerovoracaceae.6       2.308298e-01 4.245136e-01 0.0665896635 2.900230e-01
#> Anaerovoracaceae.7       1.424962e-02 1.099546e-01 0.0082299513 7.885668e-02
#> Family                   2.016848e-04 1.203348e-02 0.0003919807 2.254527e-02
#> Family.1                 2.190318e-03 2.715868e-02 0.0043109362 2.379067e-02
#> Family.2                 1.510197e-01 4.232456e-01 0.0506603269 4.355520e-01
#> Family.3                 6.183268e-03 4.190082e-02 0.0003919807 3.090402e-02
#> Peptostreptococcaceae    1.815441e-02 4.673724e-02 0.0003919807 2.992432e-02
#> Peptostreptococcaceae.1  5.637217e-02 1.749733e-01 0.0162626108 1.188758e-01
#> Peptostreptococcaceae.2  1.426974e-02 6.505801e-02 0.0003919806 4.562458e-02
#> Peptostreptococcaceae.3  1.843321e-01 3.944149e-01 0.0599641922 2.638962e-01
#> Peptostreptococcaceae.4  2.823067e-02 3.646173e-02 0.0003919806 2.310366e-02
#> X.25                     1.842930e-02 4.910074e-02 0.0003919806 3.708818e-02
#> Selenomonadaceae         1.313653e-01 2.397646e-01 0.0043292836 1.748917e-01
#> Selenomonadaceae.1       2.464576e-01 4.620441e-01 0.0201180184 3.697643e-01
#> Veillonellaceae          5.933785e-02 1.473375e-01 0.0081980873 8.790663e-02
#> Veillonellaceae.1        3.990520e-02 1.286295e-01 0.0042941805 7.547553e-02
#> Veillonellaceae.2        1.458185e-01 2.708433e-01 0.0082568170 1.474719e-01
#> Veillonellaceae.3        3.128286e-01 5.431961e-01 0.0609864325 3.020263e-01
#> Veillonellaceae.4        2.024962e+00 4.496995e+00 0.1206376849 9.553831e-01
#> Pelospora                2.016848e-04 2.592083e-03 0.0003919807 8.118744e-03
#> X.26                     1.616064e-02 6.229547e-02 0.0003919807 3.708818e-02
#> Fusobacterium            1.136707e+00 4.568650e+00 0.0449353328 6.931691e-01
#> X.27                     6.782298e-01 1.621516e+00 0.1145188044 4.841000e-01
#> Leptotrichia             5.810071e-01 1.537132e+00 0.0786406994 5.489465e-01
#> Streptobacillus          1.285240e-01 2.288028e-01 0.0003919806 1.205550e-01
#> SR1                      1.223982e-01 2.378828e-01 0.0003919806 1.845246e-01
#> SR1.1                    1.082580e-01 1.894837e-01 0.0082023864 1.759434e-01
#> oral.1                   2.394386e-02 4.718335e-02 0.0003919806 3.101471e-02
#> P22                      1.214577e-02 4.977710e-03 0.0003919807 2.262661e-02
#> X.28                     1.788469e-01 3.987915e-01 0.0341741427 4.460290e-01
#> Saccharibacteria         5.340241e-02 2.123081e-01 0.0124246161 2.247808e-01
#> X.29                     3.752500e-02 1.422936e-01 0.0082891368 1.845110e-01
#> Candidatus               1.833965e-01 4.497985e-01 0.0295407069 3.627111e-01
#> TM7a                     4.962555e-02 1.227060e-01 0.0043379752 1.501289e-01
```

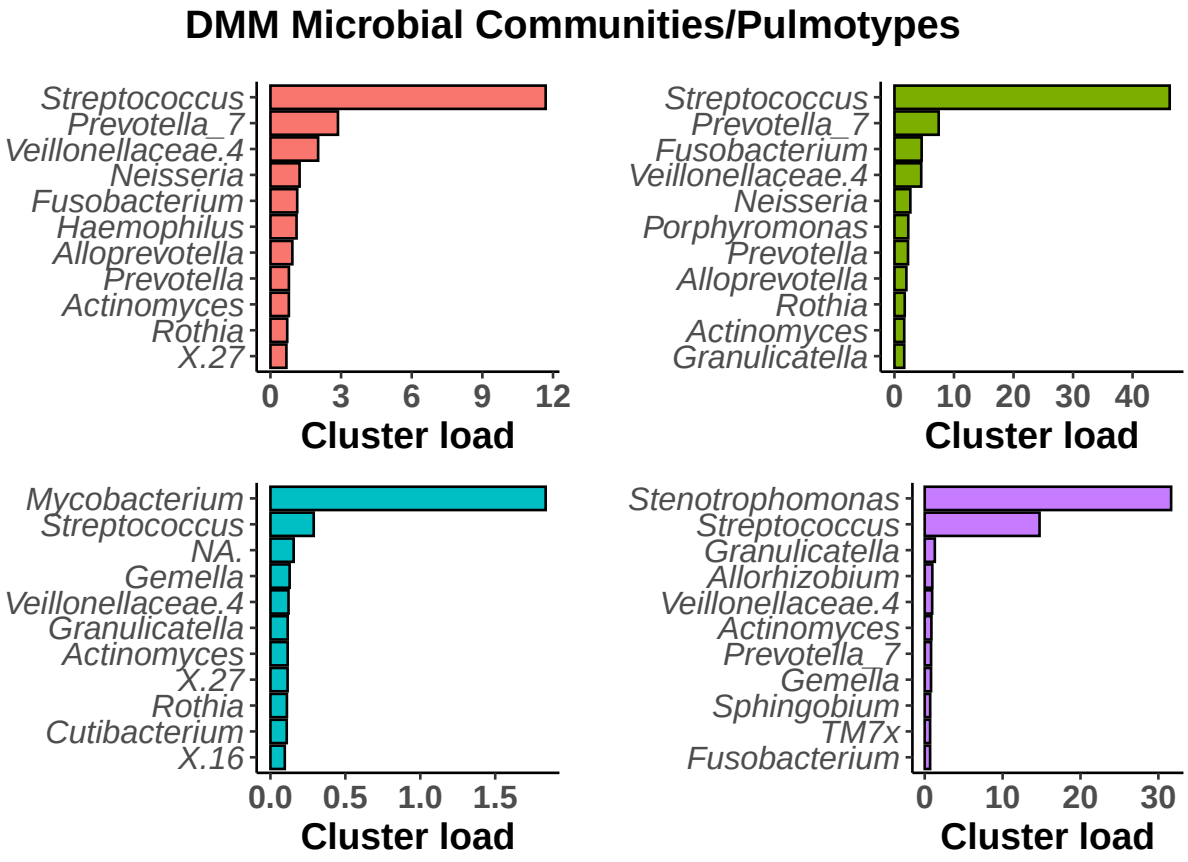
```
#> TM7x                3.248882e-01 7.802154e-01 0.0732848421 6.935257e-01
#> uncultured.1        9.634432e-02 2.386447e-01 0.0083549465 2.425989e-01
#> Candidatus.1        1.415331e-02 4.571265e-02 0.0043250332 6.650069e-02
#> X.30                4.170729e-03 2.669885e-02 0.0003919807 2.262661e-02
#> X.31                4.171927e-03 4.710506e-02 0.0003919807 6.756070e-02
#> uncultured.2        2.198373e-03 3.161448e-02 0.0043512535 1.289874e-01
#> X.32                2.393422e-02 7.325279e-02 0.0282982364 2.084332e-01
#> Brevundimonas       2.353583e-02 6.249622e-02 0.0201747668 1.310571e-01
#> Caulobacter         2.106875e-02 4.023012e-02 0.0043268760 8.145892e-02
#> Reyranella          6.154343e-03 4.927169e-03 0.0281561028 7.224186e-04
#> X.33                6.178179e-03 3.703161e-02 0.0126867540 1.146034e-01
#> Bosea               4.186633e-03 2.433249e-02 0.0251083924 1.212464e-01
#> X.34                2.016848e-04 1.457469e-02 0.0003919807 3.879208e-02
#> Allorhizobium       5.654496e-02 1.374722e-01 0.0206892365 9.753471e-01
#> Mesorhizobium       1.846500e-02 5.271568e-02 0.0243324622 8.186471e-02
#> Bradyrhizobium      1.226067e-02 4.520906e-02 0.0278341598 7.529095e-02
#> X.35                1.817546e-02 1.197786e-02 0.0003919807 7.942454e-03
#> Sphingobium         3.904228e-02 8.693335e-02 0.0253257000 7.036454e-01
#> Sphingomonas        3.972066e-02 1.481584e-01 0.0245796935 3.256535e-01
#> Lautropia           1.643041e-01 2.400766e-01 0.0082568170 1.245719e-01
#> Ralstonia           2.457171e-02 5.094801e-02 0.0862435719 8.521599e-02
#> X.36                4.169935e-03 7.321760e-03 0.0484734371 7.224176e-04
#> Aquabacterium       2.233949e-03 1.916242e-02 0.0240022803 7.942454e-03
#> Comamonas           2.518267e-02 4.167248e-02 0.0003919806 2.335821e-02
#> Methylibium         1.027372e-02 7.214561e-03 0.0796405206 7.216079e-04
#> X.37                1.370872e-01 2.264664e-01 0.0042941805 1.057277e-01
#> Alysiella           2.657029e-02 3.150178e-02 0.0003919806 7.224252e-04
#> Eikenella           6.088523e-02 8.828579e-02 0.0003919806 3.002911e-02
#> Kingella            4.889417e-02 1.126081e-01 0.0042941805 3.809204e-02
#> Neisseria           1.237702e+00 2.669119e+00 0.0509425940 5.599895e-01
#> Methyloversatilis   1.438784e-02 2.474509e-02 0.0083607236 4.698932e-02
#> Propionivibrio      4.138051e-03 7.345153e-03 0.0003919806 1.516395e-02
#> Cardiobacterium     4.355621e-02 9.220202e-02 0.0003919806 6.867935e-02
#> Enterobacter        1.464927e-02 2.202603e-02 0.0359570060 2.992432e-02
#> Escherichia         1.883998e-02 3.796072e-02 0.0399689062 4.017233e-02
#> Kosakonia           4.230058e-03 4.972712e-03 0.0121554883 8.093261e-03
#> Providencia         2.220810e-03 2.338345e-04 0.0003919807 7.224259e-04
#> Actinobacillus      1.436242e-01 1.770229e-01 0.0043109362 1.004470e-01
#> Aggregatibacter     2.903764e-01 4.406827e-01 0.0003919806 1.614566e-01
#> Haemophilus         1.109845e+00 1.629506e+00 0.0408937242 4.668268e-01
#> Acinetobacter       4.195538e-03 2.338443e-04 0.0617785129 7.224162e-04
#> Faucicola           2.686307e-02 2.862736e-02 0.0003919806 1.555382e-02
#> Moraxella           4.314832e-02 9.480875e-02 0.0043193810 4.088550e-02
#> Pseudomonas         1.276204e-02 1.440581e-02 0.0406484968 3.024403e-02
#> Stenotrophomonas    8.210283e-02 1.841535e-01 0.0291864826 3.163731e+01
#> X.38                3.712654e-02 1.575401e-01 0.0003919806 5.588156e-02
#> X.39                2.016848e-04 9.738553e-03 0.0003919807 7.224263e-04
#> Treponema           1.123997e-01 2.900150e-01 0.0082460411 1.687477e-01
#> Fretibacterium      4.151036e-02 1.179165e-01 0.0003919806 9.353079e-02
#> Pyramidobacter      2.213480e-03 2.632764e-02 0.0003919807 3.059986e-02
#> X.40                2.185975e-03 7.296069e-03 0.0043016451 2.981714e-02
#> Dioszegia           2.185975e-03 7.296069e-03 0.0003919807 7.224263e-04
#> Vishniacozyma       8.169151e-03 1.684443e-02 0.0160658612 1.538274e-02
```

```
#> NA.                    3.588294e-02 8.202643e-02 0.1549463592 3.974559e-01

# Extract fitted values from the models
p1 <- fitted(dmn_list[[1]], scale = TRUE)  # Fitted values from the first model
p5 <- fitted(best_fit, scale = TRUE)  # Fitted values from the best model

# Compute the mean difference
meandiff <- colSums(abs(p5 - as.vector(p1)))

# Display the mean difference
print(meandiff)
#> [1] 0.4344662 0.6288278 0.9956112 0.8636098

# Extract the fitted values from the best model
x <- mixture(best_fit)

# Initialize an empty list to store plots
plot_list <- vector("list", ncol(fitted(best_fit)))

# Loop through each cluster
for (k in seq(ncol(fitted(best_fit)))) {
  # Melt the fitted values to long format
  d <- melt(fitted(best_fit))
  colnames(d) <- c("OTU", "cluster", "value")  # Rename columns
  # Filter and process data for the current cluster
  d <- subset(d, cluster == k) %>%
    arrange(value) %>%
    mutate(OTU = factor(OTU, levels = unique(OTU))) %>%
    filter(abs(value) > quantile(abs(value), 0.8))  # Keep top 20% most significant OTUs

  # Define a function to generate color hues
  gg_color_hue <- function(n) {
    hues = seq(15, 375, length = n + 1)
    hcl(h = hues, l = 65, c = 100)[1:n]
  }

  # Get colors for the bars
  cols = gg_color_hue(ncol(fitted(best_fit)))

  # Create the plot for the current cluster
  p <- ggplot(d[(length(d$value) - 10):length(d$value), ],
              aes(x = OTU, y = value)) +
    xlab("") +  # Remove x-axis label
    ylab("Cluster load") +  # Label y-axis
    geom_bar(stat = "identity", fill = cols[k], colour = "black") +  # Bar plot with color and border
    coord_flip() +  # Flip coordinates for horizontal bars
    theme_classic() +  # Apply classic theme
    theme(axis.text.y = element_text(face = "italic"),  # Style y-axis text
          axis.text = element_text(size = 13, face = 'bold'),  # Style axis text
          axis.title = element_text(size = 15, face = 'bold'))  # Style axis titles

  # Store the plot in the list
  plot_list[[k]] <- p
```

```
}

# Combine and display all plots in plot_list
combined_plot<- grid.arrange(grobs = plot_list, ncol = 2)
```




```
# Create the combined plot with cowplot and add a title
combined_plot <- ggdraw() +
  draw_plot(combined_plot, 0, 0, 1, 0.9) +  # Adjust plot size to leave space for title
  draw_label("DMM Microbial Communities/Pulmotypes",
             x = 0.5, y = 0.95, hjust = 0.5, size = 16, fontface = "bold")
print(combined_plot)
```



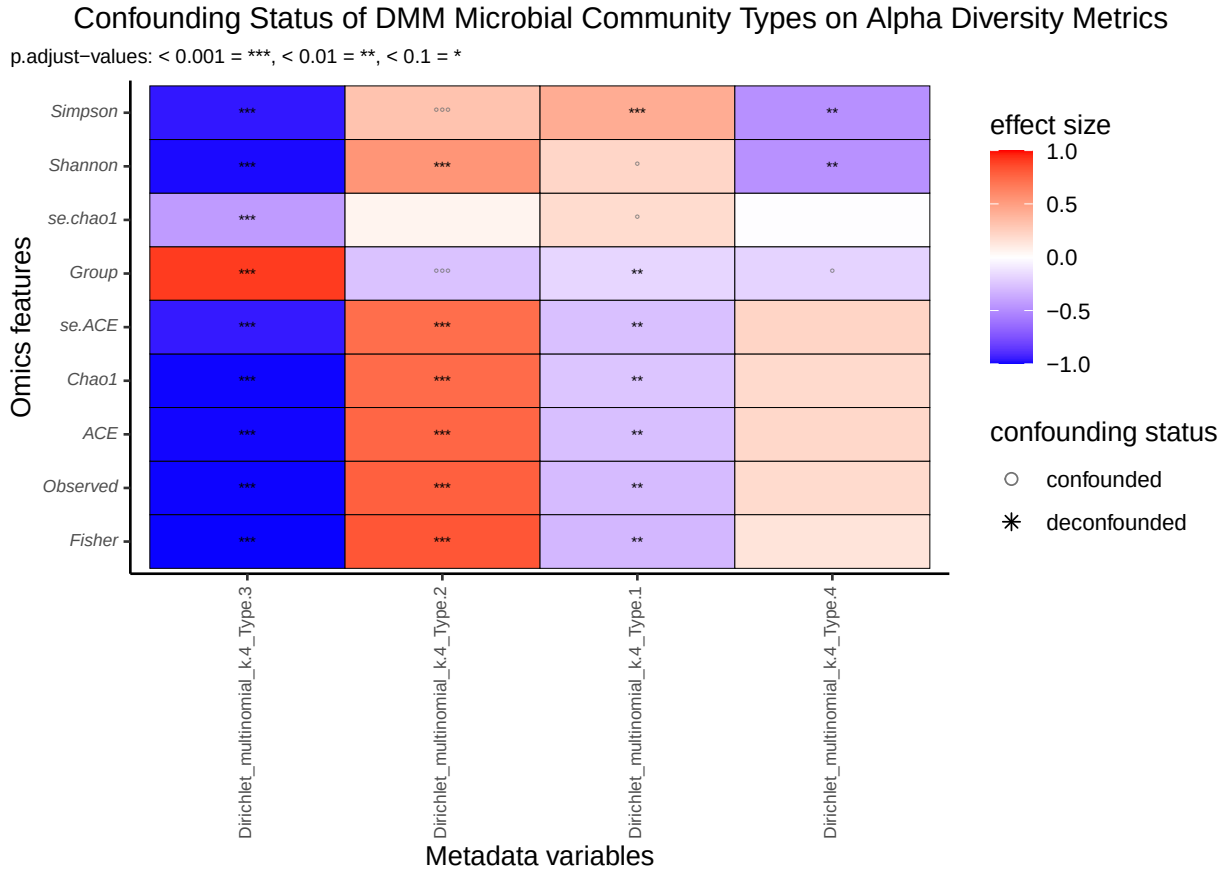

```
ggsave("Results/Figure 3.jpg", plot = combined_plot, dpi = 600, width = 12, height = 8)
```

Cofounding factors between microbiome alpha diversity metrics and the different DMM microbial communities

```
alpha_features <- alpha_metrics_df_Group


meta_alpha_decon <- Genexpert_metadata_df

# Fit Dirichlet Multinomial models with components ranging from 1 to all_dmns
dmn_list <- lapply(1:all_dmns, function(k) dmn(as.matrix(t(genus_table)), k, verbose = FALSE))

# Determine the best model (based on Laplace)
best_fit_index <- best_lplc_index
best_fit <- dmn_list[[best_fit_index]]

# Assign clusters for the best fit Dirichlet model
cluster_result <- as.data.frame(mixture(best_fit, assign = TRUE))
cluster_col_name <- paste0("Dirichlet_multinomial_k=", best_fit_index)
colnames(cluster_result) <- cluster_col_name
```

```r
# Add the cluster results to meta_alpha_decon
meta_alpha_decon <- cbind(meta_alpha_decon, cluster_result)

# Update cluster labels to descriptive names
cluster_levels <- unique(meta_alpha_decon[[cluster_col_name]])
cluster_labels <- paste("Type", cluster_levels)
meta_alpha_decon[[cluster_col_name]] <- factor(
  meta_alpha_decon[[cluster_col_name]],
  levels = cluster_levels,
  labels = cluster_labels
)

# Add a new column 'smpl' with row names
meta_alpha_decon$smpl <- row.names(meta_alpha_decon)

# Create dummy columns for the selected cluster column
meta_alpha_decon <- fastDummies::dummy_cols(.data = meta_alpha_decon, select_columns = cluster_col_name

# Set the row names of 'meta_alpha_decon' to the 'smpl' column and clean up
row.names(meta_alpha_decon) <- meta_alpha_decon$smpl
meta_alpha_decon$smpl <- NULL
meta_alpha_decon[[cluster_col_name]] <- NULL

# Order the dataframes by row names
alpha_features <- alpha_features[order(rownames(alpha_features)), ]
meta_alpha_decon <- meta_alpha_decon[order(rownames(meta_alpha_decon)), ]
# Recode the values in the Group column in both dataframes
meta_alpha_decon$Group[meta_alpha_decon$Group == "Xpert-ve"] <- 0
meta_alpha_decon$Group[meta_alpha_decon$Group == "Xpert+ve"] <- 1

# Replace values in Group column
alpha_features$Group <- ifelse(alpha_features$Group == "Xpert-ve", 0,
                               ifelse(alpha_features$Group == "Xpert+ve", 1, alpha_features$Grou

#Run metadecofound function
meta_alpha_out <- metadeconfoundR::MetaDeconfound(
  featureMat = alpha_features,
  metaMat = meta_alpha_decon,
  nnodes = 4,  # Number of nodes to use for parallel processing
  logfile = here::here("MetadeconfoundR_feature_alpha.log")  # Path to the log file
)
#Heatmap
alpha_metaDR <- metadeconfoundR::BuildHeatmap(
  meta_alpha_out,
  d_col = c("blue", "white", "red"),  # Color scheme for the heatmap
  d_range = "full"  # Use the full range of the data for color scaling
)
alpha_metaDR1 <- alpha_metaDR +
  theme(axis.text.y = element_text(face ="italic"))
# Add a title to the alpha_metaDR1 plot
alpha_metaDR1 <- alpha_metaDR1 +
  ggtitle("Confounding Status of DMM Microbial Community Types on Alpha Diversity Metrics") +
  theme(plot.title = element_text(hjust = 0.5, size = 12))  # Center and size the title
```

```
print(alpha_metaDR1)
```



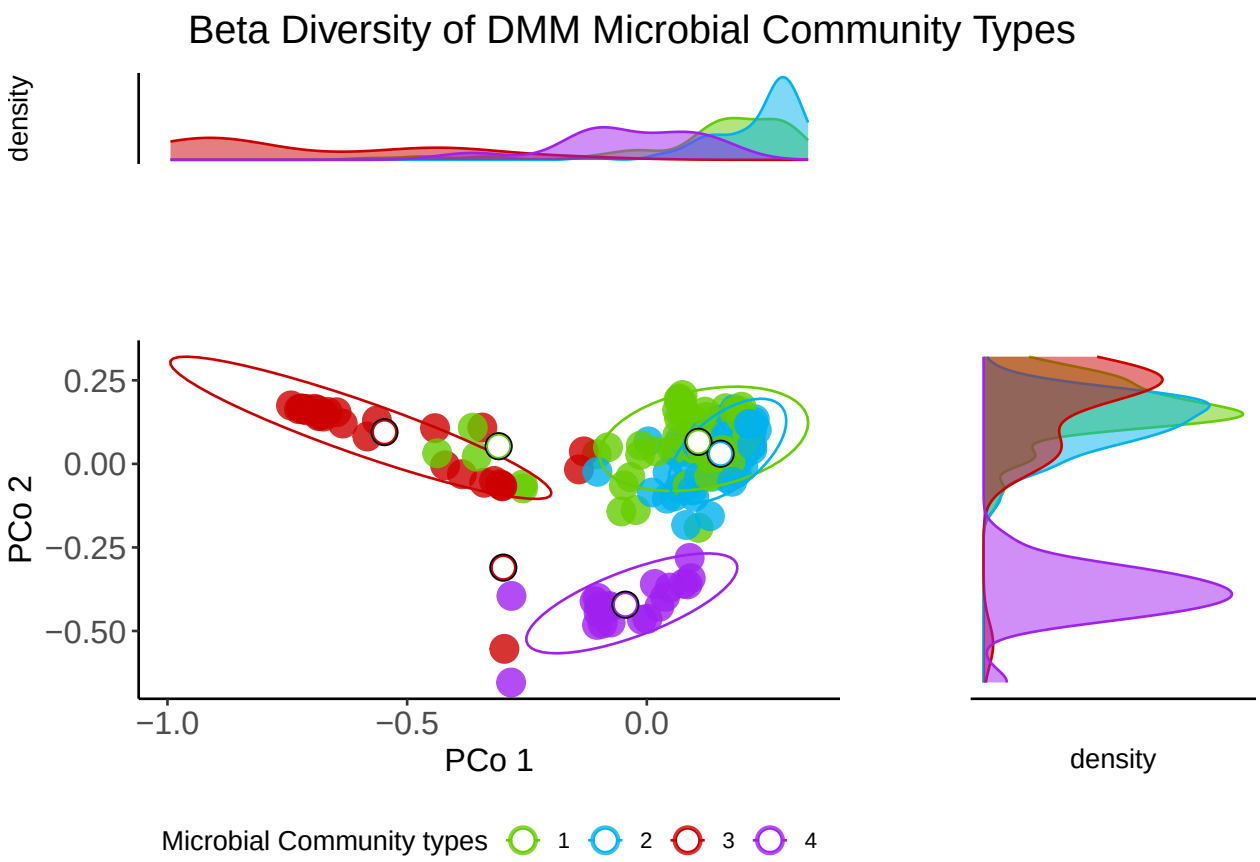

```
ggsave("Results/Figure 4.jpg",alpha_metaDR1, width = 8, height =8,limitsize = FALSE, dpi=600)
```

Beta diversity of the different DMM microbial community types/pulmotypes

```
# Create cluster results and combine with metadata

# Add the cluster results to metadata_beta
metadata_beta <- cbind(Group_metadata_beta, cluster_result)
metadata_beta[[cluster_col_name]] <- as.character(metadata_beta[[cluster_col_name]])

# Calculate centroids for the clusters
centroids <- aggregate(cbind(V1, V2) ~ ., data = metadata_beta, FUN = mean)

# Define custom colors for the plot
ComCol <- c("chartreuse3", "deepskyblue2", "red3", "purple","orange","violet")

# Create the main plot with points and ellipses
Bcluster <- metadata_beta %>%
```

```
  ggplot(aes(x = V1, y = V2, color = .data[[cluster_col_name]])) +
  theme_classic() +
  scale_color_manual(values = ComCol) +
  geom_point(size = 5, alpha = 0.8) +
  xlab("PCo 1") + ylab("PCo 2") +
  labs(color = "Microbial Community types") +
  theme(axis.title.x = element_text(size = 13),
        axis.text.x = element_text(size = 13),
        axis.text.y = element_text(size = 13),
        axis.title.y = element_text(size = 13),
        legend.position = "bottom") +
  stat_ellipse() +
  geom_point(data = centroids, size = 5, shape = 16, color = "black") +
  geom_point(data = centroids, size = 4, shape = 21, fill = "white")

#Create the x-axis density plot
xdensity <- metadata_beta %>%
  ggplot(aes(x = V1, fill = .data[[cluster_col_name]], color = .data[[cluster_col_name]])) +
  geom_density(alpha = .5) +
  scale_fill_manual(values = ComCol) +
  scale_color_manual(values = ComCol) +
  theme_classic() +
  theme(axis.title.x = element_blank(),
        axis.text.x = element_blank(),
        axis.ticks.x = element_blank(),
        axis.line.x = element_blank(),
        axis.text.y = element_blank(),
        axis.ticks.y = element_blank(),
        legend.position = "none")

#Create the y-axis density plot with flipped coordinates
ydensity <- metadata_beta %>%
  ggplot(aes(x = V2, fill = .data[[cluster_col_name]], color = .data[[cluster_col_name]])) +
  geom_density(alpha = .5) +
  scale_fill_manual(values = ComCol) +
  scale_color_manual(values = ComCol) +
  theme_classic() +
  theme(axis.text.x = element_blank(),
        axis.ticks.x = element_blank(),
        axis.title.y = element_blank(),
        axis.text.y = element_blank(),
        axis.ticks.y = element_blank(),
        axis.line.y = element_blank(),
        legend.position = "none") +
  coord_flip()

#Create a blank plot for layout purposes
blankPlot <- ggplot() +
  geom_blank(aes(1, 1)) +
  theme_void()

#Combine the plots into a single grid layout
Beta_diversity <-
```

```r
  cowplot::plot_grid(
    xdensity + theme(plot.margin = unit(c(0, 0, 0, 0), "cm")),
    blankPlot + theme(plot.margin = unit(c(0, 0, 0, 0), "cm")),
    Bcluster + theme(legend.position = "bottom",
                     plot.margin = unit(c(0, 0, 0, 0), "cm")),
    ydensity + theme(plot.margin = unit(c(0, 0, 0, 0), "cm")),
    nrow = 2,
    rel_widths = c(1, 0.5),
    rel_heights = c(0.5, 1),
    align = "hv"
  )
# Add a title to the Beta_diversity plot
Beta_diversity <- cowplot::ggdraw() +
  cowplot::draw_plot(Beta_diversity, 0, 0, 1, 0.9) +  # Adjust plot position and size
  cowplot::draw_label("Beta Diversity of DMM Microbial Community Types",
                      x = 0.5, y = 0.95, hjust = 0.5, size = 16)

# Display the combined plot
print(Beta_diversity)
```




```r
ggsave("Results/Figure 5.jpg",Beta_diversity, width = 8, height =8,limitsize = FALSE, dpi=600)
```

Relative abundance

Phylum

```
# Custom theme for plots
custom_theme <- ggplot2::theme_bw() +
  ggplot2::theme(
    panel.background = ggplot2::element_blank(),  # Remove background color
    panel.grid = ggplot2::element_blank(),  # Remove grid lines
    legend.position = "right",  # Position legend on the right
    legend.title = ggplot2::element_blank(),  # Remove legend title
    legend.background = ggplot2::element_blank(),  # Remove legend background
    legend.key = ggplot2::element_blank(),  # Remove legend key background
    plot.title = ggplot2::element_text(vjust = -8.5, hjust = 0.1),  # Adjust plot title position
    axis.title.y = ggplot2::element_text(colour = "black"),  # Set Y axis title color
    axis.text = ggplot2::element_text()  # Set axis text style
  )
# Color palette for plots
color_set <- c(RColorBrewer::brewer.pal(11, "Set1"), RColorBrewer::brewer.pal(9, "Pastel1"))
# Assign the phyloseq object to a variable
phyloseq_object <- MtbInfectionStatus_phyloseq
group_col <- "Group"  # Define the group column name
rank <- "Phylum"  # Define the taxonomic rank
add_labels <- TRUE  # Flag to add labels
show_sd <- FALSE  # Flag to show standard deviation
top_n <- 10  # Number of top taxa to include
transform_abundance <- TRUE  # Flag to transform data to relative abundance

# Get unique levels for group column
axis_order <- phyloseq::sample_data(phyloseq_object)$Group %>% unique()

# Perform taxonomic glomming (grouping)
phyloseq_data <- ggClusterNet::tax_glom_wt(ps = phyloseq_object, ranks = rank)

# Transform to relative abundance if specified
if (transform_abundance == TRUE) {
  phyloseq_data <- phyloseq_data %>%
    phyloseq::transform_sample_counts(function(x) {x / sum(x)})
}

# Extract OTU and taxonomy tables
otu_table <- phyloseq::otu_table(phyloseq_data)
tax_table <- phyloseq::tax_table(phyloseq_data)

# Adjust taxonomy table to group non-top taxa as "others"
for (i in 1:dim(tax_table)[1]) {
  if (row.names(tax_table)[i] %in% names(sort(rowSums(otu_table), decreasing = TRUE)[1:top_n])) {
    tax_table[i, rank] <- tax_table[i, rank]
  } else {
    tax_table[i, rank] <- "others"
  }
}
# Update the phyloseq object with the modified taxonomy table
```

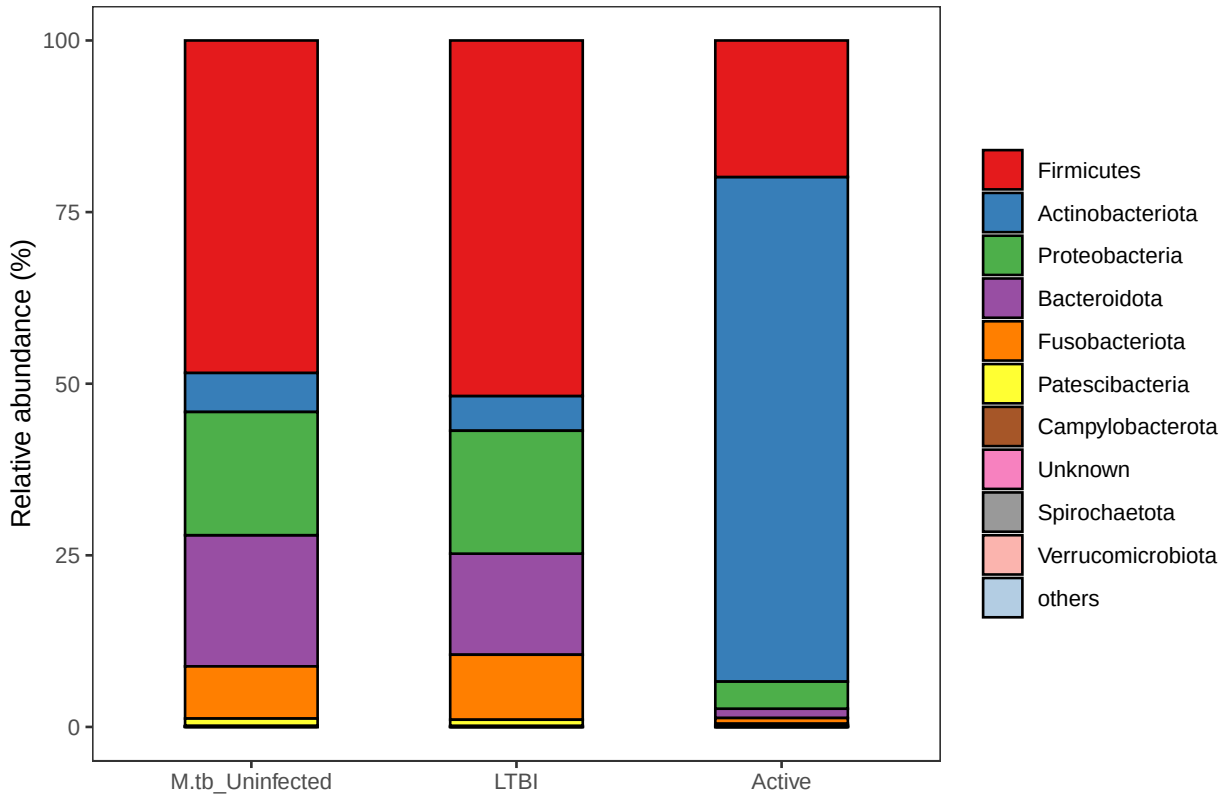
```r
phyloseq::tax_table(phyloseq_data) <- tax_table

# Melt the phyloseq object to a data frame
taxonomies <- phyloseq_data %>%
  phyloseq::psmelt()

# Convert abundance to percentage
taxonomies$Abundance <- taxonomies$Abundance * 100
# Rename taxonomy column to "taxonomy"
colnames(taxonomies) <- gsub(rank, "taxonomy", colnames(taxonomies))

# Initialize an empty data frame
final_data <- c()
# Loop over each group to calculate relative abundance
for (i in 1:length(unique(phyloseq::sample_data(phyloseq_object)$Group))) {
  group_name <- as.data.frame(table(phyloseq::sample_data(phyloseq_object)$Group))[i, 1]
  group_size <- as.data.frame(table(phyloseq::sample_data(phyloseq_object)$Group))[i, 2]

  # Filter data by group
  group_data <- taxonomies %>%
    dplyr::filter(Group == group_name)
  group_data$Abundance <- group_data$Abundance / group_size

  # Create a temporary data frame for the current group
  temp_data <- data.frame(Sample = group_data$Sample,
                          Abundance = group_data$Abundance,
                          taxonomy = group_data$taxonomy,
                          Group = group_data$Group)

  # Combine data for all groups
  if (i == 1) {
    final_data <- temp_data
  } else {
    final_data <- rbind(final_data, temp_data)
  }
}

# Update taxonomies data frame with combined data
taxonomies <- final_data

# Group data by taxonomy and group columns
grouped_taxa <- dplyr::group_by(taxonomies, taxonomy, Group)
# Summarize abundance and standard deviation by group and taxonomy
summarized_data <- dplyr::summarize(grouped_taxa, sum(Abundance), sd(Abundance))

# Group data by taxonomy to calculate total abundance
taxonomy_groups <- dplyr::group_by(taxonomies, taxonomy)
total_abundance <- dplyr::summarize(taxonomy_groups, sum(Abundance))
head(total_abundance)
#> # A tibble: 6 x 2
#>   taxonomy         `sum(Abundance)`
#>   <chr>                       <dbl>
#> 1 Actinobacteriota             84.3
```

```
#> 2 Bacteroidota               35.2
#> 3 Campylobacterota            0.272
#> 4 Firmicutes                120.
#> 5 Fusobacteriota             17.9
#> 6 Patescibacteria             2.19
# Rename columns for total abundance data
colnames(total_abundance) <- c("taxonomy", "total_sum")
# Arrange taxa by total abundance in descending order
total_abundance <- dplyr::arrange(total_abundance, desc(total_sum))

# Preview the summarized data
head(summarized_data)
#> # A tibble: 6 x 4
#> # Groups:   taxonomy [2]
#>   taxonomy         Group           `sum(Abundance)` `sd(Abundance)`
#>   <chr>            <chr>                      <dbl>           <dbl>
#> 1 Actinobacteriota Active                     73.5            1.08
#> 2 Actinobacteriota LTBI                        5.07           0.382
#> 3 Actinobacteriota M.tb_Uninfected             5.70           0.124
#> 4 Bacteroidota     Active                      1.36           0.153
#> 5 Bacteroidota     LTBI                       14.7            1.26
#> 6 Bacteroidota     M.tb_Uninfected            19.1            0.355
# Rename columns in summarized data
colnames(summarized_data) <- c("taxonomy", "group", "Abundance", "sd")
# Convert taxonomy to a factor and order by total abundance
summarized_data$taxonomy <- factor(summarized_data$taxonomy, order = TRUE, levels = total_abundance$taxo

# Copy summarized data for further processing
summarized_data_2 <- summarized_data

# Calculate cumulative sums for plotting labels
plot_data <- plyr::ddply(summarized_data_2, "group", summarize, label_sd = cumsum(Abundance), label_y =
head(plot_data)
#>    group label_sd  label_y
#> 1 Active 73.51348 36.75674
#> 2 Active 74.87137 74.19242
#> 3 Active 74.88777 74.87957
#> 4 Active 94.77396 84.83087
#> 5 Active 95.60601 95.18999
#> 6 Active 95.89413 95.75007

# Combine summarized data with cumulative sum labels
plot_data <- cbind(as.data.frame(summarized_data_2), as.data.frame(plot_data)[, -1])

# Set label column to be the taxonomy
plot_data$label <- plot_data$taxonomy

# Order taxonomy levels by total abundance
plot_data$taxonomy <- factor(plot_data$taxonomy, order = TRUE, levels = c(as.character(total_abundance$t

# Create a bar plot for relative abundance
bar_plot <- ggplot(plot_data, aes(x = group, y = Abundance, fill = taxonomy, order = taxonomy)) +
  geom_bar(stat = "identity", width = 0.5, color = "black") +
```

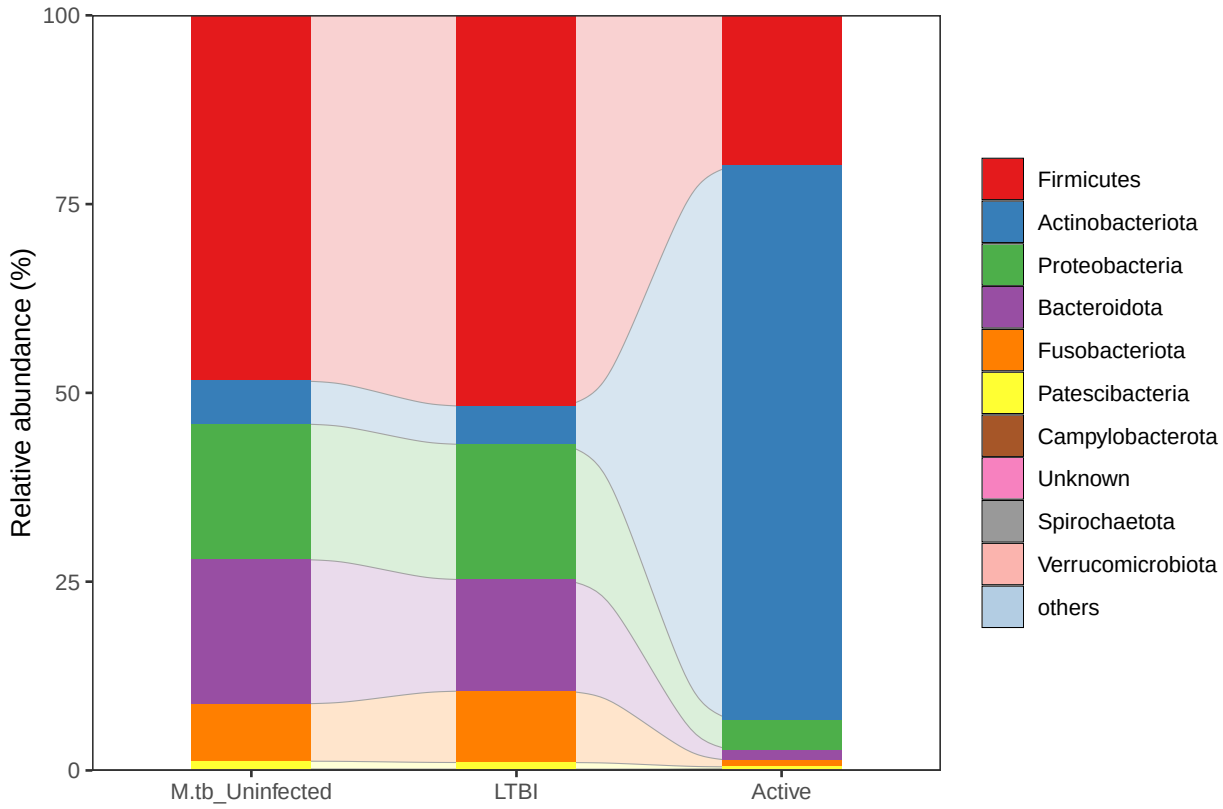
```
  theme(axis.title.x = element_blank()) +
  theme(legend.text = element_text(size = 6)) +
  scale_y_continuous(name = "Relative abundance (%)") +
  guides(fill = guide_legend(title = rank)) +
  labs(x = "", y = "Relative abundance (%)", title = "")

# Adjust X-axis to follow the order of groups
bar_plot <- bar_plot + scale_x_discrete(limits = axis_order)

# Add error bars if the show_sd flag is TRUE
if (show_sd == TRUE) {
  bar_plot <- bar_plot +
    geom_errorbar(aes(ymin = label_sd - sd, ymax = label_sd + sd), width = 0.2)
}

# Convert sample data to a data frame
sample_data <- as.data.frame(phyloseq::sample_data(phyloseq_object))
# Adjust text angle on the X-axis if there are more than 3 groups
if (length(unique(sample_data$Group)) > 3) {
  bar_plot <- bar_plot + theme(axis.text.x = element_text(angle = 45, vjust = 1, hjust = 1))
}

# Get the taxonomy levels
taxa <- plot_data$taxonomy
# Calculate the number of factor levels
num_factors <- taxa %>% levels() %>% length()

# Summarize the number of occurrences of each factor level
factor_summary <- taxa %>%
  as.factor() %>%
  summary() %>%
  as.data.frame()

# Add row names as an ID column
factor_summary$id <- row.names(factor_summary)

# Arrange factors by ID and data by taxonomy
arranged_factors <- dplyr::arrange(factor_summary, id)
arranged_plot_data <- dplyr::arrange(plot_data, taxonomy)
head(arranged_plot_data)
#>           taxonomy           group Abundance        sd  label_sd   label_y
#> 1       Firmicutes          Active 19.886191 0.9281756  99.99162 99.991625
#> 2       Firmicutes            LTBI 51.785083 2.5829114 100.00000 99.995812
#> 3       Firmicutes M.tb_Uninfected 48.417191 0.4710692   5.06555  2.532775
#> 4 Actinobacteriota          Active 73.513479 1.0768376  73.51348 36.756740
#> 5 Actinobacteriota            LTBI  5.065550 0.3823114  74.87137 74.192424
#> 6 Actinobacteriota M.tb_Uninfected  5.702155 0.1238589  74.88777 74.879569
#>              label
#> 1       Firmicutes
#> 2       Firmicutes
#> 3       Firmicutes
#> 4 Actinobacteriota
#> 5 Actinobacteriota
```

```
#> 6 Actinobacteriota
# Assign an ID to each row in the arranged plot data
arranged_plot_data$ID <- factor(rep(c(1:num_factors), factor_summary$.))

head(arranged_plot_data)
#>           taxonomy           group Abundance        sd  label_sd   label_y
#> 1       Firmicutes          Active 19.886191 0.9281756  99.99162 99.991625
#> 2       Firmicutes            LTBI 51.785083 2.5829114 100.00000 99.995812
#> 3       Firmicutes M.tb_Uninfected 48.417191 0.4710692   5.06555  2.532775
#> 4 Actinobacteriota          Active 73.513479 1.0768376  73.51348 36.756740
#> 5 Actinobacteriota            LTBI  5.065550 0.3823114  74.87137 74.192424
#> 6 Actinobacteriota M.tb_Uninfected  5.702155 0.1238589  74.88777 74.879569
#>              label ID
#> 1       Firmicutes  1
#> 2       Firmicutes  1
#> 3       Firmicutes  1
#> 4 Actinobacteriota  2
#> 5 Actinobacteriota  2
#> 6 Actinobacteriota  2
arranged_plot_data$Abundance
#>  [1] 19.886191413 51.785083383 48.417190971 73.513479241  5.065550426
#>  [6]  5.702155363  3.943758692 17.908110417 17.947841619  1.357889101
#> [11] 14.722943432 19.116239320  0.832052493  9.472137561  7.595283834
#> [16]  0.288115774  0.871687299  1.032733374  0.016401643  0.146890748
#> [21]  0.108970667  0.151190044  0.005484613  0.016627884  0.002546348
#> [26]  0.012671308  0.029314897  0.000000000  0.000000000  0.029325513
#> [31]  0.008375249  0.009440813  0.004316559

# Create an alluvial plot for relative abundance
alluvial_plot <- ggplot(arranged_plot_data, aes(x = group, y = Abundance, fill = taxonomy, alluvium = ta
  ggalluvial::geom_flow(aes(fill = taxonomy, colour = taxonomy),
            stat = "alluvium", lode.guidance = "rightleft",
            color = "black", size = 0.2, width = 0.35, alpha = .2)  +
  geom_bar(width = 0.45, stat = "identity") +
  labs(x = "", y = "Relative abundance (%)", title = "") +
  guides(fill = guide_legend(title = rank), color = FALSE) +
  scale_y_continuous(expand = c(0, 0))

# Add error bars to the alluvial plot if the show_sd flag is TRUE
if (show_sd == TRUE) {
  alluvial_plot <- alluvial_plot +
    geom_errorbar(aes(ymin = label_sd - sd, ymax = label_sd + sd), width = 0.2)
}

# Adjust text angle on the X-axis if there are more than 3 groups
if (length(unique(sample_data$Group)) > 3) {
  alluvial_plot <- alluvial_plot + theme(axis.text.x = element_text(angle = 45, vjust = 1, hjust = 1))
}

# Get rank names from the phyloseq object
phyloseq::rank_names(phyloseq_object)
#> [1] "Kingdom" "Phylum"  "Class"   "Order"   "Family"  "Genus"   "Species"
```

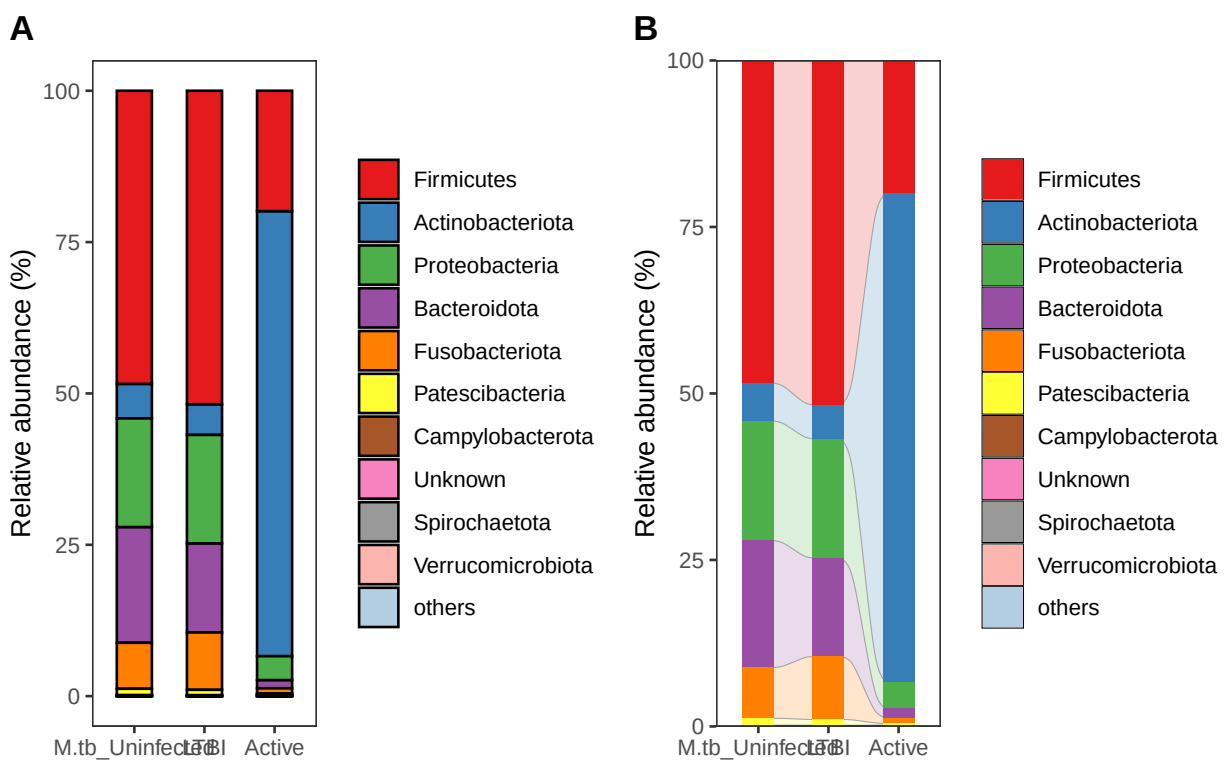
```
# Customize the bar plot with a manual color scale and theme
bar_plot_1 <- bar_plot +
  scale_fill_manual(values = color_set) +
  scale_x_discrete(limits = axis_order) +
  custom_theme
bar_plot_1
```




```
# Customize the alluvial plot with a manual color scale and theme
bar_plot_2 <- alluvial_plot +
  scale_fill_manual(values = color_set) +
  scale_x_discrete(limits = axis_order) +
  custom_theme
bar_plot_2
```




```
# Combine the bar plot and alluvial plot side by side using cowplot
combined_plot <- cowplot::plot_grid(bar_plot_1, bar_plot_2, labels = c("A", "B"))

# Add a title to the combined plot
combined_plot <- cowplot::ggdraw() +
  cowplot::draw_plot(combined_plot, 0, 0, 1, 0.9) +  # Adjust plot position and size
  cowplot::draw_label("Phylum Relative Abundance According to Mtb Infection Status",
                      x = 0.5, y = 0.95, hjust = 0.5, size = 16)

# Print the combined plot with title
print(combined_plot)
```




```
# Save the combined plot as a high-resolution JPG file
ggsave("Results/Figure 6A.jpg", plot = combined_plot, width = 12, height = 6, dpi = 600)
```

Genus

```
# Assign the phyloseq object to a variable
phyloseq_object <- MtbInfectionStatus_phyloseq
group_col <- "Group"  # Define the group column name
rank <- "Genus"  # Define the taxonomic rank
add_labels <- TRUE  # Flag to add labels
show_sd <- FALSE  # Flag to show standard deviation
top_n <- 10  # Number of top taxa to include
transform_abundance <- TRUE  # Flag to transform data to relative abundance

# Get unique levels for group column
axis_order <- phyloseq::sample_data(phyloseq_object)$Group %>% unique()

# Perform taxonomic glomming (grouping)
phyloseq_data <- ggClusterNet::tax_glom_wt(ps = phyloseq_object, ranks = rank)

# Transform to relative abundance if specified
```

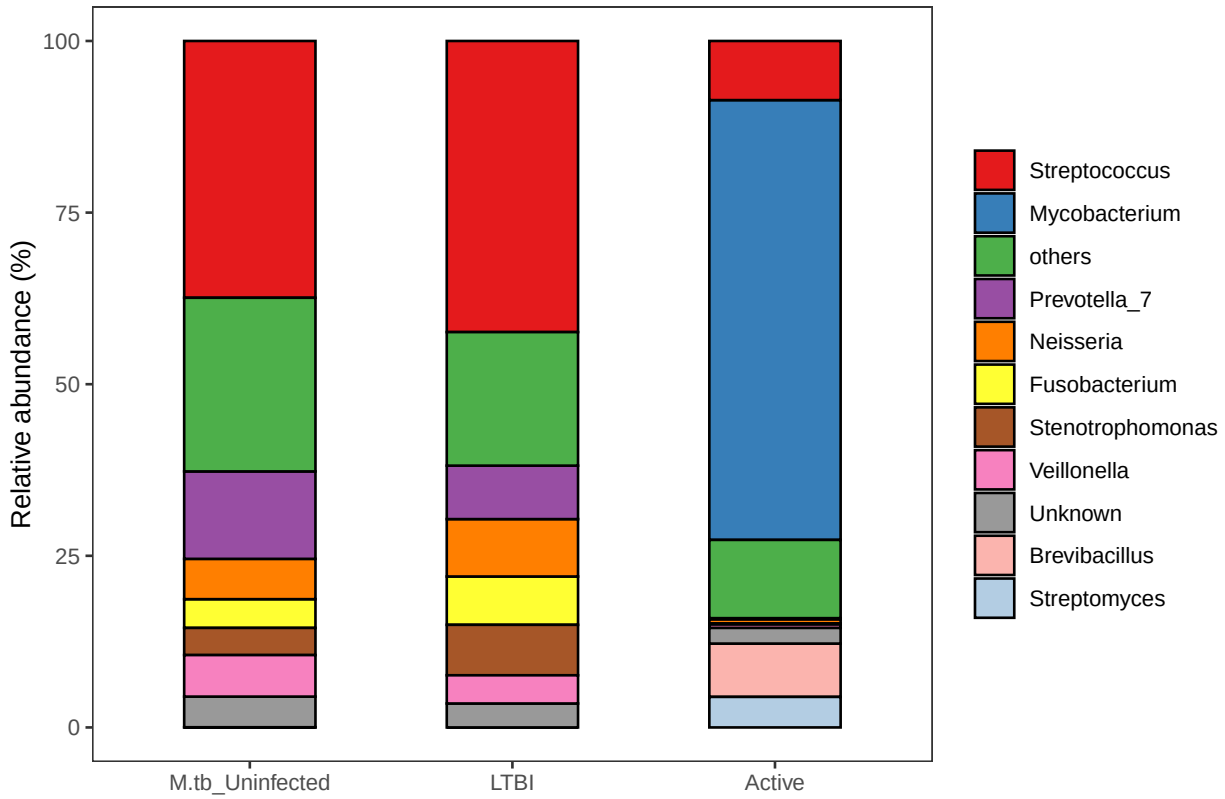
```r
if (transform_abundance == TRUE) {
  phyloseq_data <- phyloseq_data %>%
    phyloseq::transform_sample_counts(function(x) {x / sum(x)})
}

# Extract OTU and taxonomy tables
otu_table <- phyloseq::otu_table(phyloseq_data)
tax_table <- phyloseq::tax_table(phyloseq_data)

# Adjust taxonomy table to group non-top taxa as "others"
for (i in 1:dim(tax_table)[1]) {
  if (row.names(tax_table)[i] %in% names(sort(rowSums(otu_table), decreasing = TRUE)[1:top_n])) {
    tax_table[i, rank] <- tax_table[i, rank]
  } else {
    tax_table[i, rank] <- "others"
  }
}
# Update the phyloseq object with the modified taxonomy table
phyloseq::tax_table(phyloseq_data) <- tax_table

# Melt the phyloseq object to a data frame
taxonomies <- phyloseq_data %>%
  phyloseq::psmelt()

# Convert abundance to percentage
taxonomies$Abundance <- taxonomies$Abundance * 100
# Rename taxonomy column to "taxonomy"
colnames(taxonomies) <- gsub(rank, "taxonomy", colnames(taxonomies))

# Initialize an empty data frame
final_data <- c()
# Loop over each group to calculate relative abundance
for (i in 1:length(unique(phyloseq::sample_data(phyloseq_object)$Group))) {
  group_name <- as.data.frame(table(phyloseq::sample_data(phyloseq_object)$Group))[i, 1]
  group_size <- as.data.frame(table(phyloseq::sample_data(phyloseq_object)$Group))[i, 2]

  # Filter data by group
  group_data <- taxonomies %>%
    dplyr::filter(Group == group_name)
  group_data$Abundance <- group_data$Abundance / group_size

  # Create a temporary data frame for the current group
  temp_data <- data.frame(Sample = group_data$Sample,
                          Abundance = group_data$Abundance,
                          taxonomy = group_data$taxonomy,
                          Group = group_data$Group)

  # Combine data for all groups
  if (i == 1) {
    final_data <- temp_data
  } else {
    final_data <- rbind(final_data, temp_data)
  }
```

```
}

# Update taxonomies data frame with combined data
taxonomies <- final_data

# Group data by taxonomy and group columns
grouped_taxa <- dplyr::group_by(taxonomies, taxonomy, Group)
# Summarize abundance and standard deviation by group and taxonomy
summarized_data <- dplyr::summarize(grouped_taxa, sum(Abundance), sd(Abundance))

# Group data by taxonomy to calculate total abundance
taxonomy_groups <- dplyr::group_by(taxonomies, taxonomy)
total_abundance <- dplyr::summarize(taxonomy_groups, sum(Abundance))
head(total_abundance)
#> # A tibble: 6 x 2
#>   taxonomy         `sum(Abundance)`
#>   <chr>                       <dbl>
#> 1 Brevibacillus                7.76
#> 2 Fusobacterium               11.4
#> 3 Mycobacterium               64.1
#> 4 Neisseria                   14.8
#> 5 Prevotella_7                20.7
#> 6 Stenotrophomonas            11.3
# Rename columns for total abundance data
colnames(total_abundance) <- c("taxonomy", "total_sum")
# Arrange taxa by total abundance in descending order
total_abundance <- dplyr::arrange(total_abundance, desc(total_sum))

# Preview the summarized data
head(summarized_data)
#> # A tibble: 6 x 4
#> # Groups:   taxonomy [2]
#>   taxonomy      Group           `sum(Abundance)` `sd(Abundance)`
#>   <chr>         <chr>                      <dbl>           <dbl>
#> 1 Brevibacillus Active                  7.76           0.769
#> 2 Brevibacillus LTBI                    0              0
#> 3 Brevibacillus M.tb_Uninfected         0.0000414      0.00000744
#> 4 Fusobacterium Active                  0.176          0.0186
#> 5 Fusobacterium LTBI                    7.04           1.15
#> 6 Fusobacterium M.tb_Uninfected         4.19           0.139
# Rename columns in summarized data
colnames(summarized_data) <- c("taxonomy", "group", "Abundance", "sd")
# Convert taxonomy to a factor and order by total abundance
summarized_data$taxonomy <- factor(summarized_data$taxonomy, order = TRUE, levels = total_abundance$taxo

# Copy summarized data for further processing
summarized_data_2 <- summarized_data

# Calculate cumulative sums for plotting labels
plot_data <- plyr::ddply(summarized_data_2, "group", summarize, label_sd = cumsum(Abundance), label_y =
head(plot_data)
#>    group  label_sd   label_y
#> 1 Active  7.764394  3.882197
```

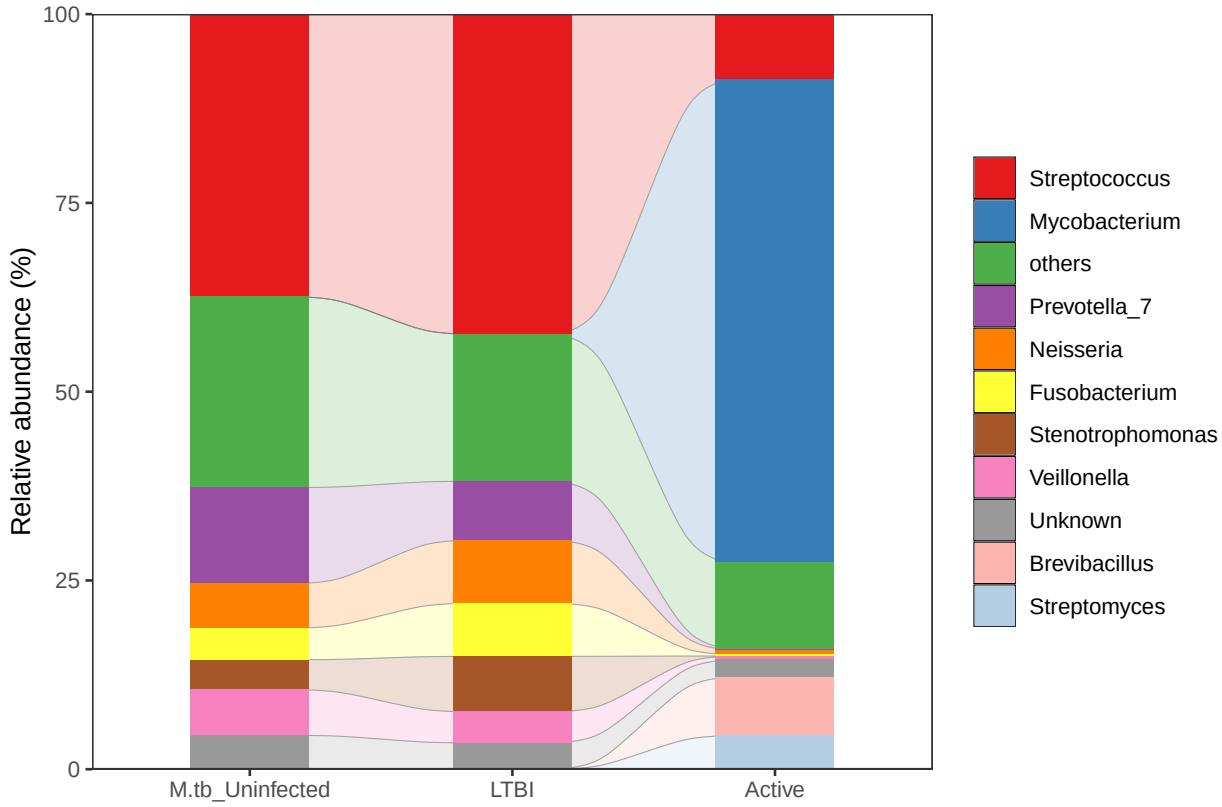
```
#> 2 Active  7.939998  7.852196
#> 3 Active 72.007652 39.973825
#> 4 Active 72.599165 72.303408
#> 5 Active 72.761836 72.680500
#> 6 Active 72.762470 72.762153

# Combine summarized data with cumulative sum labels
plot_data <- cbind(as.data.frame(summarized_data_2), as.data.frame(plot_data)[, -1])

# Set label column to be the taxonomy
plot_data$label <- plot_data$taxonomy

# Order taxonomy levels by total abundance
plot_data$taxonomy <- factor(plot_data$taxonomy, order = TRUE, levels = c(as.character(total_abundance$

# Create a bar plot for relative abundance
bar_plot <- ggplot(plot_data, aes(x = group, y = Abundance, fill = taxonomy, order = taxonomy)) +
  geom_bar(stat = "identity", width = 0.5, color = "black") +
  theme(axis.title.x = element_blank()) +
  theme(legend.text = element_text(size = 6)) +
  scale_y_continuous(name = "Relative abundance (%)") +
  guides(fill = guide_legend(title = rank)) +
  labs(x = "", y = "Relative abundance (%)", title = "")

# Adjust X-axis to follow the order of groups
bar_plot <- bar_plot + scale_x_discrete(limits = axis_order)

# Add error bars if the show_sd flag is TRUE
if (show_sd == TRUE) {
  bar_plot <- bar_plot +
    geom_errorbar(aes(ymin = label_sd - sd, ymax = label_sd + sd), width = 0.2)
}

# Convert sample data to a data frame
sample_data <- as.data.frame(phyloseq::sample_data(phyloseq_object))
# Adjust text angle on the X-axis if there are more than 3 groups
if (length(unique(sample_data$Group)) > 3) {
  bar_plot <- bar_plot + theme(axis.text.x = element_text(angle = 45, vjust = 1, hjust = 1))
}

# Get the taxonomy levels
taxa <- plot_data$taxonomy
# Calculate the number of factor levels
num_factors <- taxa %>% levels() %>% length()

# Summarize the number of occurrences of each factor level
factor_summary <- taxa %>%
  as.factor() %>%
  summary() %>%
  as.data.frame()

# Add row names as an ID column
factor_summary$id <- row.names(factor_summary)
```

```r
# Arrange factors by ID and data by taxonomy
arranged_factors <- dplyr::arrange(factor_summary, id)
arranged_plot_data <- dplyr::arrange(plot_data, taxonomy)
head(arranged_plot_data)
#>        taxonomy           group    Abundance           sd label_sd  label_y
#> 1 Streptococcus          Active 8.614375e+00 5.469314e-01 72.95060 72.95043
#> 2 Streptococcus            LTBI 4.240755e+01 2.700678e+00 76.43687 74.69373
#> 3 Streptococcus M.tb_Uninfected 3.740700e+01 4.756377e-01 80.54534 78.49111
#> 4 Mycobacterium          Active 6.406765e+01 1.247160e+00 81.37685 77.06966
#> 5 Mycobacterium            LTBI 6.984606e-04 2.328202e-04 85.82627 83.60156
#> 6 Mycobacterium M.tb_Uninfected 4.826456e-04 3.789528e-05 88.12495 86.97561
#>           label
#> 1 Streptococcus
#> 2 Streptococcus
#> 3 Streptococcus
#> 4 Mycobacterium
#> 5 Mycobacterium
#> 6 Mycobacterium
# Assign an ID to each row in the arranged plot data
arranged_plot_data$ID <- factor(rep(c(1:num_factors), factor_summary$.))

head(arranged_plot_data)
#>        taxonomy           group    Abundance           sd label_sd  label_y
#> 1 Streptococcus          Active 8.614375e+00 5.469314e-01 72.95060 72.95043
#> 2 Streptococcus            LTBI 4.240755e+01 2.700678e+00 76.43687 74.69373
#> 3 Streptococcus M.tb_Uninfected 3.740700e+01 4.756377e-01 80.54534 78.49111
#> 4 Mycobacterium          Active 6.406765e+01 1.247160e+00 81.37685 77.06966
#> 5 Mycobacterium            LTBI 6.984606e-04 2.328202e-04 85.82627 83.60156
#> 6 Mycobacterium M.tb_Uninfected 4.826456e-04 3.789528e-05 88.12495 86.97561
#>           label ID
#> 1 Streptococcus  1
#> 2 Streptococcus  1
#> 3 Streptococcus  1
#> 4 Mycobacterium  2
#> 5 Mycobacterium  2
#> 6 Mycobacterium  2
arranged_plot_data$Abundance
#>  [1] 8.614375e+00 4.240755e+01 3.740700e+01 6.406765e+01 6.984606e-04
#>  [6] 4.826456e-04 1.139800e+01 1.945466e+01 2.529569e+01 1.626713e-01
#> [11] 7.788218e+00 1.273785e+01 5.915123e-01 8.364471e+00 5.872532e+00
#> [16] 1.756048e-01 7.036583e+00 4.189324e+00 6.342343e-04 7.352734e+00
#> [21] 3.957739e+00 4.770567e-01 4.108475e+00 6.049974e+00 2.298674e+00
#> [26] 3.486270e+00 4.459788e+00 7.764394e+00 0.000000e+00 4.141968e-05
#> [31] 4.449428e+00 3.458604e-04 2.958449e-02

# Create an alluvial plot for relative abundance
alluvial_plot <- ggplot(arranged_plot_data, aes(x = group, y = Abundance, fill = taxonomy, alluvium = ta
  ggalluvial::geom_flow(aes(fill = taxonomy, colour = taxonomy),
            stat = "alluvium", lode.guidance = "rightleft",
            color = "black", size = 0.2, width = 0.35, alpha = .2)  +
  geom_bar(width = 0.45, stat = "identity") +
  labs(x = "", y = "Relative abundance (%)", title = "") +
  guides(fill = guide_legend(title = rank), color = FALSE) +
```

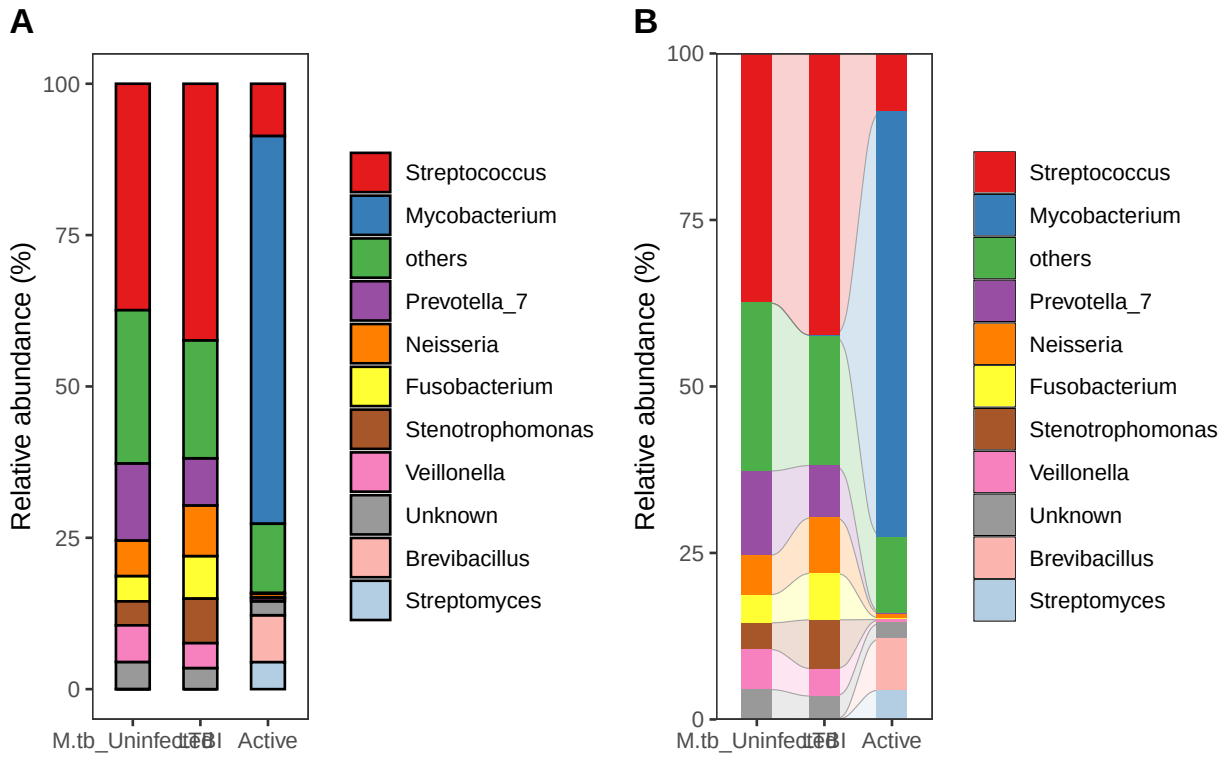
```r
  scale_y_continuous(expand = c(0, 0))

# Add error bars to the alluvial plot if the show_sd flag is TRUE
if (show_sd == TRUE) {
  alluvial_plot <- alluvial_plot +
    geom_errorbar(aes(ymin = label_sd - sd, ymax = label_sd + sd), width = 0.2)
}

# Adjust text angle on the X-axis if there are more than 3 groups
if (length(unique(sample_data$Group)) > 3) {
  alluvial_plot <- alluvial_plot + theme(axis.text.x = element_text(angle = 45, vjust = 1, hjust = 1))
}

# Get rank names from the phyloseq object
phyloseq::rank_names(phyloseq_object)
#> [1] "Kingdom" "Phylum"  "Class"   "Order"   "Family"  "Genus"   "Species"

# Customize the bar plot with a manual color scale and theme
bar_plot_1 <- bar_plot +
  scale_fill_manual(values = color_set) +
  scale_x_discrete(limits = axis_order) +
  custom_theme
bar_plot_1
```



```r
# Customize the alluvial plot with a manual color scale and theme
bar_plot_2 <- alluvial_plot +
  scale_fill_manual(values = color_set) +
  scale_x_discrete(limits = axis_order) +
  custom_theme
bar_plot_2
```




```r
# Combine the bar plot and alluvial plot side by side using cowplot
combined_plot <- cowplot::plot_grid(bar_plot_1, bar_plot_2, labels = c("A", "B"))

# Add a title to the combined plot
combined_plot <- cowplot::ggdraw() +
  cowplot::draw_plot(combined_plot, 0, 0, 1, 0.9) +  # Adjust plot position and size
  cowplot::draw_label("Genus Relative Abundance According to Mtb Infection Status",
                      x = 0.5, y = 0.95, hjust = 0.5, size = 16)

# Print the combined plot with title
print(combined_plot)
```




```
# Save the combined plot as a high-resolution JPG file
ggsave("Results/Figure 6B.jpg", plot = combined_plot, width = 12, height = 6, dpi = 600)
```

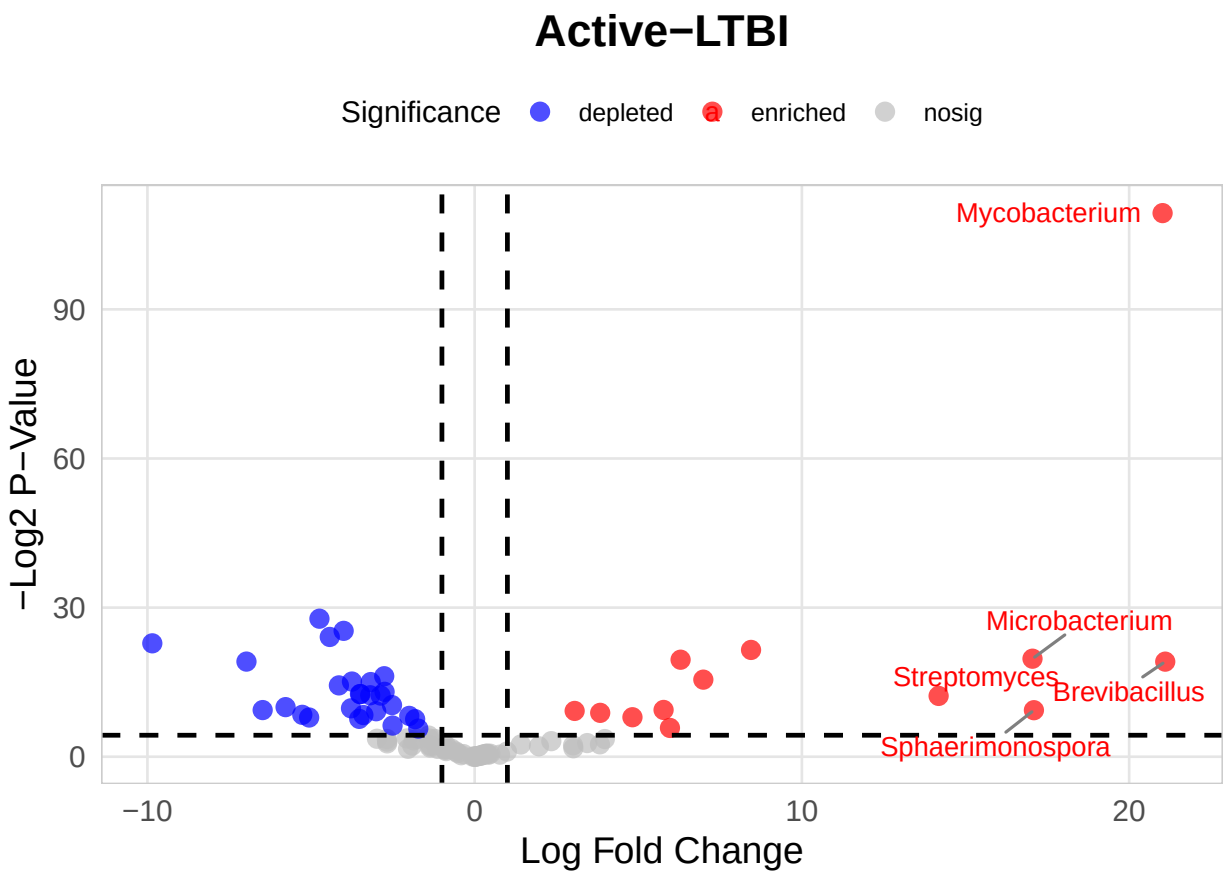
Differential abundance

```
# Load phyloseq object and process taxonomy
physeq_data <- MtbInfectionStatus_phyloseq
# Analysis parameters
analysis_group <- "Group"  # Group variable for analysis
p_value_threshold <- 0.05  # Threshold for p-value significance
log_fold_change_threshold <- 0  # Threshold for log fold change
artificial_groups <- NULL  # Placeholder for artificial group comparisons
normalization_method <- "TMM"  # Method for normalization
rank_selection <- 6  # Taxonomic rank for analysis
contrast_matrix <- NULL  # Placeholder for contrast matrix

# Initialize a list to store plots
plot_list <- list()  # List to store individual plots

# Prepare phyloseq data based on rank selection
```

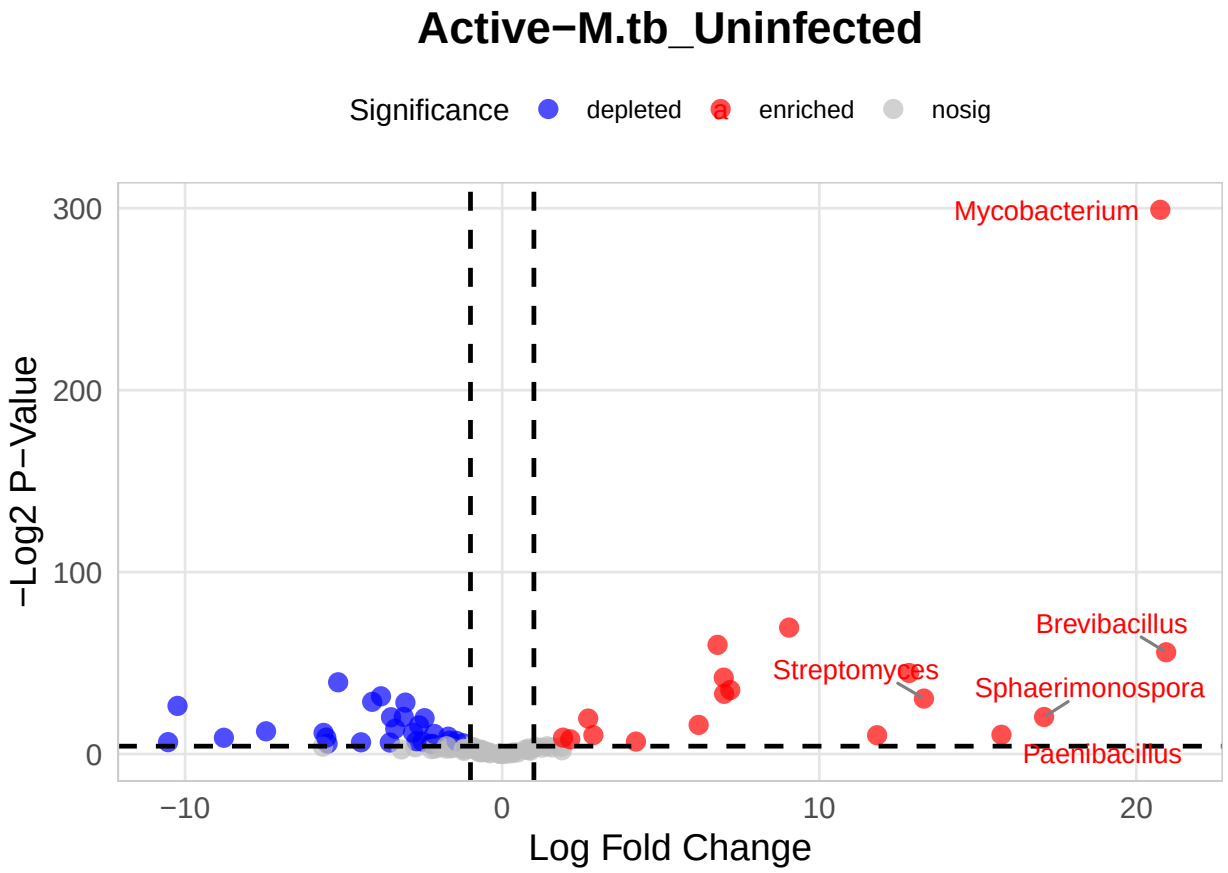
```r
if (rank_selection %in% c("OTU", "gene", "meta")) {
    physeq_data <- physeq_data  # No change needed
} else if (rank_selection %in% c(1:7)) {
    physeq_data <- physeq_data %>%
        ggClusterNet::tax_glom_wt(ranks = rank_selection)  # Aggregate taxa at specified rank
} else if (rank_selection %in% c("Kingdom", "Phylum", "Class", "Order", "Family", "Genus", "Species")) {
    # No action needed for standard taxonomic ranks
} else {
    print("Unknown rank_selection, please check")  # Print error if rank selection is invalid
}

# Prepare design matrix and count data
sample_data <- as.data.frame(phyloseq::sample_data(physeq_data))  # Convert sample data to dataframe
descriptive_groups <- as.character(levels(as.factor(sample_data$Group)))  # Extract group levels

if (is.null(artificial_groups)) {
    contrast_combinations <- combn(descriptive_groups, 2)  # Generate all pairwise contrasts
} else if (!is.null(artificial_groups)) {
    contrast_combinations <- as.matrix(contrast_matrix)  # Use provided contrast matrix
}

otu_table_data <- as.data.frame(ggClusterNet::vegan_otu(physeq_data))  # Extract OTU table
count_matrix <- as.matrix(otu_table_data)  # Convert to matrix and transpose
count_matrix <- t(count_matrix)
sample_data$SampleType <- as.factor(sample_data$Group)  # Convert Group to factor
dge_list <- edgeR::DGEList(counts = count_matrix, group = sample_data$SampleType)  # Create DGEList obj
dge_list <- edgeR::calcNormFactors(dge_list, method = normalization_method)  # Normalize counts

# Define design matrix for GLM
design_matrix <- model.matrix(~ 0 + dge_list$samples$group)  # Create design matrix without intercept
colnames(design_matrix) <- levels(sample_data$SampleType)  # Set column names
dge_list <- edgeR::estimateGLMCommonDisp(dge_list, design_matrix)  # Estimate common dispersion
dge_list <- edgeR::estimateGLMTagwiseDisp(dge_list, design_matrix)  # Estimate tagwise dispersion
glm_fit <- edgeR::glmFit(dge_list, design_matrix)  # Fit GLM

# Perform differential analysis and plot results
for (i in 1:dim(contrast_combinations)[2]) {
    comparison_groups <- contrast_combinations[, i]  # Extract current comparison groups
    print(comparison_groups)  # Print comparison groups

    contrast_name <- paste(comparison_groups[1], comparison_groups[2], sep = "-")  # Create contrast na
    contrast_matrix <- limma::makeContrasts(contrasts = contrast_name, levels = c(as.character(levels(a
    glm_lrt <- edgeR::glmLRT(glm_fit, contrast = contrast_matrix)  # Perform likelihood ratio test

    diff_test_result <- edgeR::decideTestsDGE(glm_lrt, adjust.method = "fdr", p.value = p_value_thresho
    summary(diff_test_result)  # Summarize differential test results
    result_table <- glm_lrt$table  # Extract results table
    result_table$sig <- diff_test_result  # Add significance results
    row.names(count_matrix)[1:6]  # Print row names for checking

    result_table <- cbind(result_table, padj =result_table$PValue)  # Adjust p-values
    enriched_taxa <- row.names(subset(result_table, sig == 1))  # Identify enriched taxa
    depleted_taxa <- row.names(subset(result_table, sig == -1))  # Identify depleted taxa
```

```
    result_table$level <- as.factor(ifelse(as.vector(result_table$sig) == 1, "enriched",
                                    ifelse(as.vector(result_table$sig) == -1, "depleted",
                                           ifelse(result_table$padj < 0.05, "unplot", "nosig"))))
    result_table <- data.frame(row.names = row.names(result_table), logFC = result_table$logFC, level =

    filtered_table <- result_table %>%
        dplyr::filter(level %in% c("enriched", "depleted", "nosig"))  # Filter based on significance

    filtered_table$Genus <- row.names(filtered_table)  # Add Genus names
    if (nrow(filtered_table) <= 1) {
        next  # Skip if no significant results
    }

    top_taxa <- filtered_table %>%
        dplyr::mutate(ord = logFC^2) %>%
        dplyr::filter(level != "nosig") %>%
        dplyr::arrange(desc(ord)) %>%
        head(n = 5)  # Select top 5 taxa

    # Store the top 5 enriched/depleted results in the list
    plot_list[[i]] <- ggplot(filtered_table, aes(x = logFC, y = -log2(p), color = level)) +
    geom_point(size = 3.5, alpha = 0.7, shape = 16) +  # Slightly larger point size with transparency
    geom_hline(yintercept = -log2(p_value_threshold), linetype = "dashed", color = 'black', size = 0.9)
    geom_vline(xintercept = c(-1, 1), linetype = "dashed", color = 'black', size = 0.9) +  # Thicker da
    ggrepel::geom_text_repel(data = top_taxa, aes(x = logFC, y = -log2(p), label = Genus),
                             size = 4, box.padding = 0.5, max.overlaps = Inf,
                             segment.color = 'grey50', segment.size = 0.6,
                             nudge_x = 0.2, nudge_y = 0.2) +  # Adjusted padding and overlap handling
    scale_color_manual(values = c("enriched" = "red", "depleted" = "blue", "nosig" = "grey")) +  # Colo
    labs(title = contrast_name, x = "Log Fold Change", y = "-Log2 P-Value", color = "Significance") +
    theme_minimal(base_size = 14) +  # Use a minimal theme for a cleaner look
    theme(
        plot.title = element_text(size = 18, face = "bold", hjust = 0.5),  # Center and bold title
        axis.title = element_text(size = 15),  # Larger axis titles
        axis.text = element_text(size = 12),  # Larger axis text
        legend.position = "top",  # Place legend at the top for better visibility
        legend.title = element_text(size = 12),  # Larger legend title
        legend.text = element_text(size = 10),  # Larger legend text
        panel.grid.major = element_line(size = 0.5, linetype = 'solid', color = "grey90"),  # Lighter g
        panel.grid.minor = element_blank(),  # Remove minor grid lines for a cleaner look
        panel.border = element_rect(color = "grey80", fill = NA, size = 0.5)  # Add border around the p
    )

    colnames(result_table) <- paste(contrast_name, colnames(result_table), sep = "")  # Rename columns

    if (i == 1) {
        combined_results <- result_table  # Initialize combined results
    } else {
        combined_results <- cbind(combined_results, result_table)  # Combine results
    }
}
#> [1] "Active" "LTBI"
```

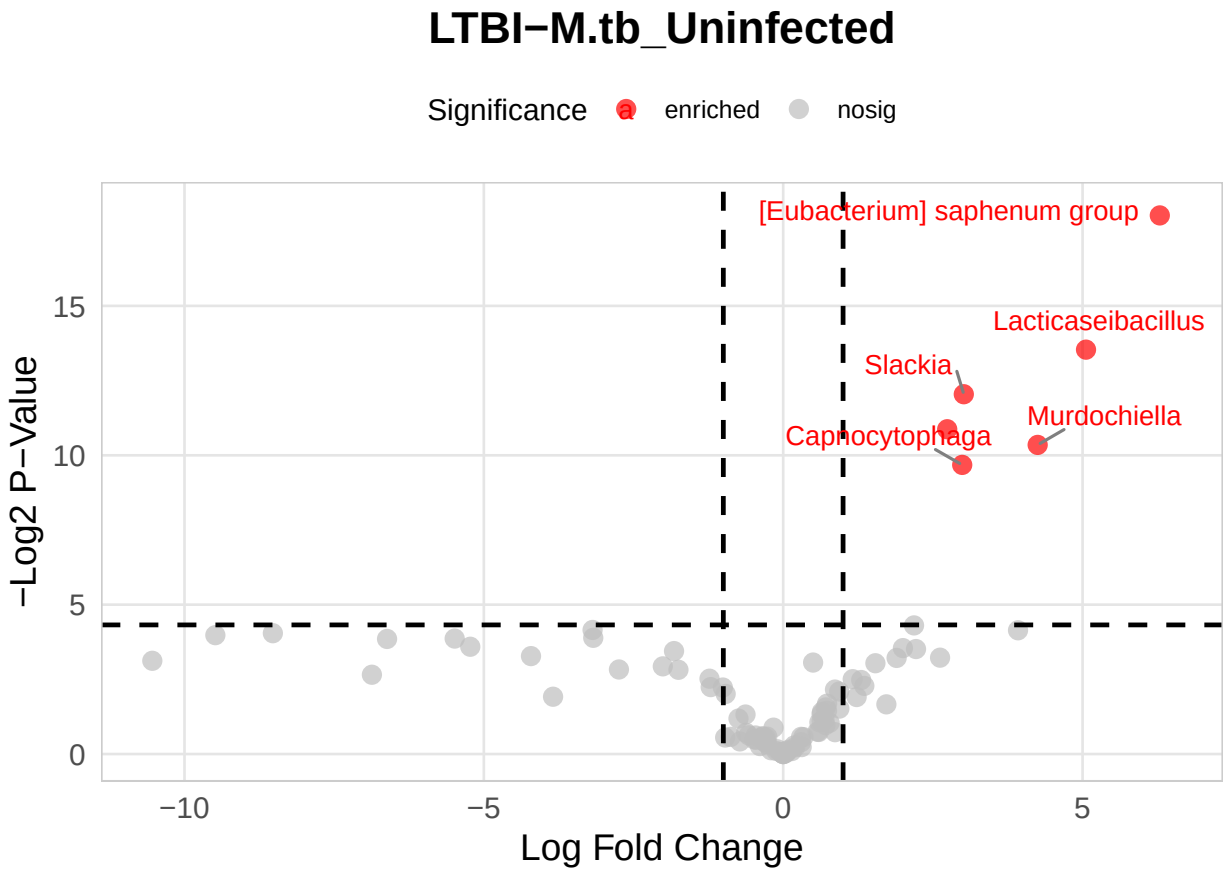
```
#> [1] "Active"          "M.tb_Uninfected"
#> [1] "LTBI"            "M.tb_Uninfected"

# Print all plots
print(plot_list)  # Print all plots stored in the list
#> [[1]]
```




```
#>
#> [[2]]
```


Active−M.tb_Uninfected
Significance
depleted
enriched
nosig
300
200
100
0
−Log2 P−Value
−10
0
10
20
Log Fold Change
Mycobacterium
Brevibacillus
Streptomyces
Sphaerimonospora
Paenibacillus

```
#>
#> [[3]]
```




```
# Combine and save the plots
combined_plot <- wrap_plots(plot_list, ncol = 3)
ggsave(filename = "Results/Figure 7.jpg", plot = combined_plot, width = 15, height = 10, dpi = 600)
```

Biomarker Identification

LEfSe analysis

```
# Load the saved phyloseq object from an RDS file
ps <- MtbInfectionStatus_phyloseq
mytheme1 <- ggplot2::theme_bw() +
  ggplot2::theme(
    panel.background = ggplot2::element_blank(),  # Remove background color
    panel.grid = ggplot2::element_blank(),  # Remove grid lines
    legend.position = "right",  # Position legend on the right
    legend.title = ggplot2::element_blank(),  # Remove legend title
    legend.background = ggplot2::element_blank(),  # Remove legend background
    legend.key = ggplot2::element_blank(),  # Remove legend key background
    plot.title = ggplot2::element_text(hjust = 0.5),  # Center plot title without vertical adjustment
    axis.title.y = ggplot2::element_text(colour = "black"),  # Set Y axis title color
    axis.text = ggplot2::element_text()  # Set axis text style
```

```
  )

# Function to generate a base plot from phyloseq data
# Function to create a circular phylogenetic tree plot with a well-edited title
p_base = function(ps, Top = 100, ranks = 6, title = "Circular Phylogenetic Tree") {
  alltax = ps %>%
    ggClusterNet::tax_glom_wt(ranks = ranks) %>%   # Collapse taxa at a given rank
    ggClusterNet::filter_OTU_ps(Top) %>%           # Filter top OTUs
    ggClusterNet::vegan_tax() %>%                  # Extract taxonomy table
    as.data.frame()                                # Convert to data frame

  alltax$OTU = row.names(alltax)                   # Add OTU IDs as a column

  # Concatenate taxonomic ranks into a single string, separated by "_Rank_"
  alltax$Kingdom = paste(alltax$Kingdom, sep = "_Rank_")
  for (i in 2:ranks) {
    alltax[, i] = paste(alltax[, i - 1], alltax[, i], sep = "_Rank_")
  }

  alltax[is.na(alltax)] = "Unknown"                # Replace NA values with "Unknown"
  trda <- MicrobiotaProcess::convert_to_treedata(alltax)  # Convert to tree data format

  # Create a circular phylogenetic tree plot
  p <- ggtree(trda, layout = "circular", size = 0.2, xlim = c(30, NA)) +
    geom_point(
      pch = 21,                                    # Set point shape
      size = 3,                                    # Set point size
      alpha = 1,                                   # Set point transparency
      fill = "#FFFFB3"                             # Set fill color for points
    ) +
    ggtitle(title) +                               # Add title with the given argument
    theme(plot.title = element_text(size = 18, face = "bold", hjust = 0.5),  # Title appearance
          axis.text = element_blank(),             # Hide axis text
          axis.title = element_blank(),            # Hide axis titles
          panel.background = element_blank(),      # Remove background grid
          panel.grid = element_blank(),            # Remove panel grid
          legend.position = "none")                # Remove legend

  # Extract the label for each node, using the last taxonomic rank in the string
  p$data$lab2 <- p$data$label %>% strsplit("_Rank_") %>%
    sapply(function(x) x[length(x)])

  # Clean up the labels
  p$data$lab2 = gsub("st__", "", p$data$lab2)
  p$data$nodeSize = 1                              # Set node size for all nodes

  return(p)                                        # Return the ggplot object
}


# Function to perform LDA (Linear Discriminant Analysis) on the phyloseq object
LDA_Micro = function(ps = ps,
                     Top = 100,
```

```
                    ranks = 6,
                    p.lvl = 0.05,
                    lda.lvl = 2,
                    seed = 11,
                    adjust.p = F) {

# Process taxonomy data similarly to p_base function
alltax = ps %>%
  ggClusterNet::tax_glom_wt(ranks = ranks) %>%
  phyloseq::filter_taxa(function(x) sum(x) > 0, TRUE) %>%
  ggClusterNet::filter_OTU_ps(Top) %>%
  ggClusterNet::vegan_tax() %>%
  as.data.frame()
alltax$OTU = row.names(alltax)

# Concatenate taxonomic ranks similarly as in p_base
alltax$Kingdom = paste(alltax$Kingdom, sep = "_Rank_")
for (i in 2:ranks) {
  alltax[, i] = paste(alltax[, i - 1], alltax[, i], sep = "_Rank_")
}

# Prepare OTU table for LDA analysis
otu = ps %>%
  ggClusterNet::tax_glom_wt(ranks = ranks) %>%
  phyloseq::filter_taxa(function(x) sum(x) > 0, TRUE) %>%
  ggClusterNet::filter_OTU_ps(Top) %>%
  ggClusterNet::vegan_otu() %>%
  t() %>%
  as.data.frame()

# Merge OTU data with taxonomy data
otu_tax = merge(otu, alltax, by = "row.names", all = F)

# Summarize OTU counts at each taxonomic level
tem = colnames(alltax)[-length(colnames(alltax))]
i = 1
tem2 = c("k__", "p__", "c__", "o__", "f__", "g__", "s__", "st__")
for (i in 1:ranks) {
  rank1 <- otu_tax %>%
    dplyr::group_by(!!sym(tem[i])) %>%
    dplyr::summarise_if(is.numeric, sum, na.rm = TRUE)
  colnames(rank1)[1] = "id"
  rank1$id = paste(tem2[i], rank1$id, sep = "")
  if (i == 1) {
    all = rank1
  }
  if (i != 1) {
    all = rbind(all, rank1)
  }
}

# Convert summarized data to phyloseq object for further analysis
data1 = as.data.frame(all)
```

```r
row.names(data1) = data1$id
data1$id = NULL

ps_G_graphlan = phyloseq::phyloseq(
  phyloseq::otu_table(as.matrix(data1), taxa_are_rows = TRUE),
  phyloseq::sample_data(ps)
)

# Extract OTU and sample data for LDA
otu = as.data.frame((ggClusterNet::vegan_otu(ps_G_graphlan)))
map = as.data.frame(phyloseq::sample_data(ps_G_graphlan))
claslbl = map$Group %>% as.factor()

set.seed(seed)  # Set seed for reproducibility

# Perform Kruskal-Wallis rank sum test on OTUs
rawpvalues <- apply(otu, 2, function(x) kruskal.test(x, claslbl)$p.value)
ord.inx <- order(rawpvalues)
rawpvalues <- rawpvalues[ord.inx]

# Adjust p-values if specified
clapvalues <- p.adjust(rawpvalues, method = "fdr")

# Prepare data for LDA
wil_datadf <- as.data.frame(otu[, ord.inx])

# Perform LDA and calculate LDAscore
ldares <- MASS::lda(claslbl ~ ., data = wil_datadf)
ldamean <- as.data.frame(t(ldares$means))
class_no <<- length(unique(claslbl))
ldamean$max <- apply(ldamean[, 1:class_no], 1, max)
ldamean$min <- apply(ldamean[, 1:class_no], 1, min)
ldamean$LDAscore <- signif(log10(1 + abs(ldamean$max - ldamean$min) / 2), digits = 3)

# Determine the class with the highest LDAscore
a = rep("A", length(ldamean$max))
for (i in 1:length(ldamean$max)) {
  name = colnames(ldamean[, 1:class_no])
  a[i] = name[ldamean[, 1:class_no][i, ] %in% ldamean$max[i]]
}
ldamean$class = a

# Add p-values and FDR to the results table
tem1 = row.names(ldamean)
ldamean$Pvalues <- signif(rawpvalues[match(row.names(ldamean), names(rawpvalues))], digits = 5)
ldamean$FDR <- signif(clapvalues, digits = 5)
resTable <- ldamean
rawNms <- rownames(resTable)
rownames(resTable) <- gsub("`", '', rawNms)

# Count significant features based on criteria
if (adjust.p) {
  de.Num <- sum(clapvalues <= p.lvl & ldamean$LDAscore >= lda.lvl)
```

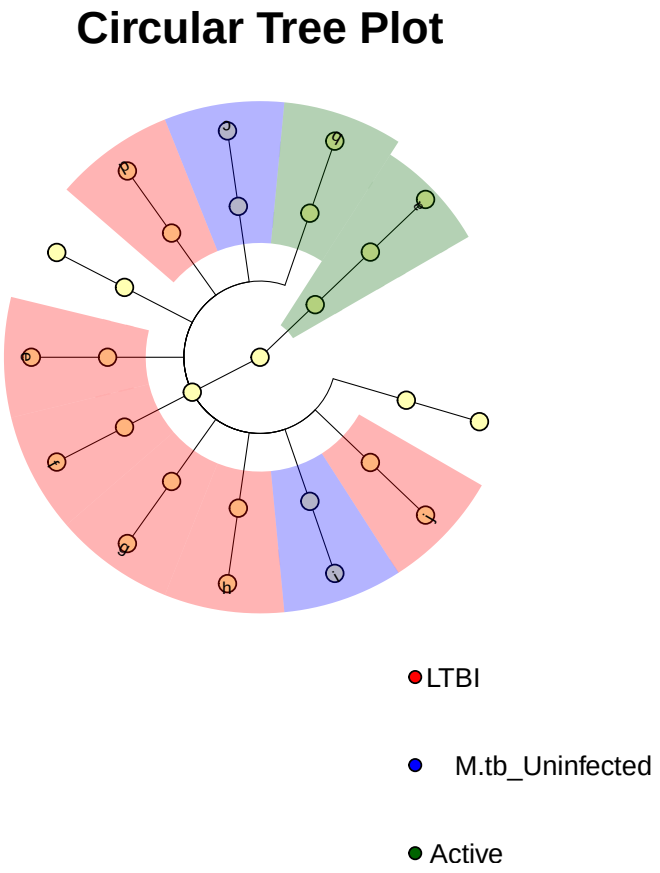
```
  } else {
    de.Num <- sum(rawpvalues <= p.lvl & ldamean$LDAscore >= lda.lvl)
  }

  # Display the results message
  if (de.Num == 0) {
    current.msg <<- "No significant features were identified with given criteria."
  } else {
    current.msg <<- paste("A total of", de.Num, "significant features with given criteria.")
  }
  print(current.msg)

  # Sort the results table by p-values and LDAscore
  ord.inx <- order(resTable$Pvalues, resTable$LDAscore)
  resTable <- resTable[ord.inx, , drop = FALSE]
  resTable <- resTable[, c(ncol(resTable), 1:(ncol(resTable) - 1))]

  # Filter the significant taxa
  if (adjust.p) {
    taxtree = resTable[clapvalues <= p.lvl & ldamean$LDAscore >= lda.lvl, ]
  } else {
    taxtree = resTable[ldamean$Pvalues <= p.lvl, ]
  }

  # Assign colors to significant taxa based on their class
  colour = c('darkgreen', 'red', "blue", "#4DAF4A", "#984EA3", "#FF7F00", "#FFFF33", "#A65628", "#F781BF
  selececol = colour[1:length(levels(as.factor(taxtree$class)))]
  names(selececol) = levels(as.factor(taxtree$class))
  A = rep("a", length(row.names(taxtree)))
  for (i in 1:length(row.names(taxtree))) {
    A[i] = selececol[taxtree$class[i]]
  }

  taxtree$color = A  # Assign color to taxtree

  # Prepare the final output as a list
  lefse_lists = data.frame(node = row.names(taxtree),
                           color = A,
                           Group = taxtree$class,
                           stringsAsFactors = FALSE)

  return(list(lefse_lists, taxtree))
}

# Function to annotate clades in the phylogenetic tree
clade.anno_wt <- function(gtree, anno.data, alpha = 0.2, anno.depth = 5, anno.x = 10,
                          anno.y = 40) {

  short.labs <- c(letters, paste(letters, 1:500, sep = ""))

  # Helper function to calculate offset for clade labels
  get_offset <- function(x) {
    (x * 0.2 + 0.2)^2
```

```
}

# Helper function to calculate the angle for clade labels
get_angle <- function(node) {
  data <- gtree$data
  sp <- tidytree::offspring(data, node)$node
  sp2 <- c(sp, node)
  sp.df <- data[match(sp2, data$node), ]
  mean(range(sp.df$angle))
}

# Arrange annotation data and assign colors to clades
anno.data <- dplyr::arrange(anno.data, node)
hilight.color <- anno.data$color
node_list <- anno.data$node
node_ids <- (gtree$data %>% filter(label %in% node_list) %>%
               arrange(label))$node
anno <- rep("yellow", nrow(gtree$data))

# Highlight clades with specified colors
for (i in 1:length(node_ids)) {
  n <- node_ids[i]
  color <- hilight.color[i]
  anno[n] <- color
  mapping <- gtree$data %>% filter(node == n)
  nodeClass <- as.numeric(mapping$nodeDepth)
  offset <- get_offset(nodeClass)
  gtree <- gtree + geom_hilight(node = n, fill = color,
                                alpha = alpha, extend = offset)
}

# Annotate clades with labels
short.labs.anno <- NULL
for (i in 1:length(node_ids)) {
  n <- node_ids[i]
  mapping <- gtree$data %>% filter(node == n)
  nodeClass <- as.numeric(mapping$nodeDepth)
  if (nodeClass <= anno.depth) {
    lab <- short.labs[1]
    short.labs <- short.labs[-1]
    if (is.null(short.labs.anno)) {
      short.labs.anno = data.frame(lab = lab, annot = mapping$lab2,
                                   stringsAsFactors = F)
    } else {
      short.labs.anno = rbind(short.labs.anno, c(lab, mapping$lab2))
    }
  } else {
    lab <- mapping$lab2
  }

  offset <- get_offset(nodeClass) - 0.4
  angle <- get_angle(n) + 90
  gtree <- gtree + geom_cladelabel(node = n, label = lab,
```

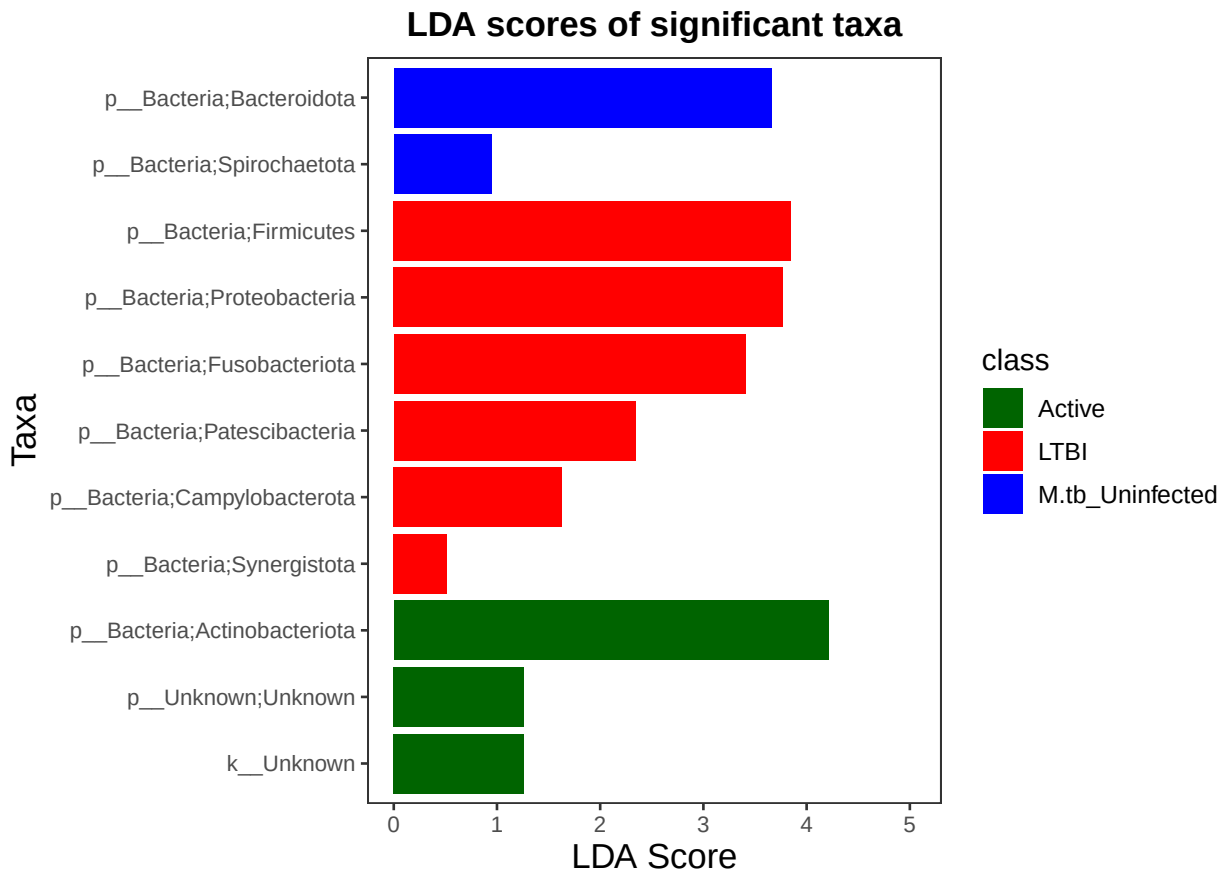
```
                                    angle = angle, fontsize = 1 + sqrt(nodeClass),
                                    offset = offset, barsize = NA, hjust = 0.5)
  }

  # Generate legend for clade colors
  if (!is.null(short.labs.anno)) {
    anno_shapes = sapply(short.labs.anno$lab, utf8ToInt)
    stable.p <- ggpubr::ggtexttable(short.labs.anno, rows = NULL,
                                    theme = ggpubr::ttheme(
                                      colnames.style = ggpubr::colnames_style(fill = "white"),
                                      tbody.style = ggpubr::tbody_style(fill = ggpubr::get_palette("RdBu
                                    ))
  }

  y = (1:length(unique(anno.data$Group)))

 pleg <- ggplot() + geom_point2(aes(
    y = y,
    x = rep(1, length(unique(anno.data$color))), fill = as.factor(1:length(unique(anno.data$Group)))
 ), pch = 21, size = 2) +
    geom_text(aes(y = y,
                  x = rep(1, length(unique(anno.data$color))), label = unique(anno.data$Group)),
              hjust = -0.2
    ) + scale_fill_manual(values = unique(anno.data$color), guide = F) +
    theme_void()


  layout <- "
  AAAAAABB
  AAAAAABB
  AAAAAABB
  CCCCCCCC
  "

  # Combine tree plot and legend layout
  if (is.null(short.labs.anno)) {
    gtree <- gtree + pleg + plot_layout(design = layout)
  } else {
    gtree <- gtree + stable.p + pleg + plot_layout(design = layout)
  }

}


# Function to create a bar plot for LEfSe results, highlighting taxa with significant LDA scores
lefse_bar = function(taxtree = tablda[[2]]) {
  taxtree = tablda[[2]]
  taxtree$ID = row.names(taxtree)

  # Prepare LEfSe data for bar plot
  taxtree$ID = gsub("_Rank_", ";", taxtree$ID)
  taxtree <- taxtree %>%
    arrange(class, LDAscore)
```

```
  taxtree$ID = factor(taxtree$ID, levels = taxtree$ID)
  taxtree$class = factor(taxtree$class, levels = unique(taxtree$class))

  # Create the bar plot with a customized title
  pbar <- ggplot(taxtree) +
  geom_bar(aes(y = ID, x = LDAscore, fill = class), stat = "identity") +
  scale_fill_manual(values = unique(taxtree$color)) +
  mytheme1 +
  scale_x_continuous(limits = c(0, max(taxtree$LDAscore) * 1.2)) +
  labs(title = "LDA scores of significant taxa",  # Add title
       x = "LDA Score", # x-axis label
       y = "Taxa") +    # y-axis label
  theme(plot.title = element_text(size = 14, face = "bold", hjust = 0.5), # Adjust title size
        axis.title.x = element_text(size = 14),
        axis.title.y = element_text(size = 14),
        legend.title = element_text(size = 12),
        legend.text = element_text(size = 10))
  return(pbar)
}


# Initialize empty lists to store the plots and results
tree_plots <- list()
bar_plots <- list()
tree_lefse_data <- list()

# Loop through different taxonomic ranks and generate plots/results
for (j in 2:6) {

  # Generate base tree plot for the given rank
  p1 <- p_base(ps, Top = 200, ranks = j,title = paste("Circular Tree Plot"))

  # Perform LDA analysis and get the results
  tablda <- LDA_Micro(ps = ps,
                      Top = 200,
                      ranks = j,
                      p.lvl = 0.05,
                      lda.lvl = 2,
                      seed = 11,
                      adjust.p = F)

  # Annotate clades in the tree plot
  p2 <- clade.anno_wt(p1, tablda[[1]], alpha = 0.3, anno.depth = 2)
  tree_plots[[paste0("Rank_", j, "_tree_plot")]] <- p2  # Save tree plot in the list

  # Create a bar plot of LEfSe results
  p <- lefse_bar(taxtree = tablda[[2]])
  bar_plots[[paste0("Rank_", j, "_bar_plot")]] <- p  # Save bar plot in the list

  # Save LEfSe data for the current rank
  res <- tablda[[2]]
  tree_lefse_data[[paste0("Rank_", j, "_tree_lefse_data")]] <- res  # Save data in the list
}
```

```
#> [1] "A total of 6 significant features with given criteria."
#> [1] "A total of 16 significant features with given criteria."
#> [1] "A total of 35 significant features with given criteria."
#> [1] "A total of 63 significant features with given criteria."
#> [1] "A total of 89 significant features with given criteria."

# Access the plots and data for rank 2
tree_plots[["Rank_2_tree_plot"]]
```




```
bar_plots[["Rank_2_bar_plot"]]
```




```
tree_lefse_data[["Rank_2_tree_lefse_data"]]
#>                                          FDR      Active         LTBI
#> p__Bacteria_Rank_Bacteroidota     5.7740e-09   420.90625  8494.111111
#> p__Bacteria_Rank_Fusobacteriota   9.2038e-09   231.53125  5374.222222
#> p__Bacteria_Rank_Campylobacterota 1.3014e-08     4.81250    88.888889
#> p__Bacteria_Rank_Proteobacteria   2.0776e-08  1883.03125 13637.888889
#> p__Bacteria_Rank_Actinobacteriota 3.7172e-08 34977.71875  2881.666667
#> k__Unknown                        9.6045e-08    35.78125     1.444444
#> p__Unknown_Rank_Unknown           9.6045e-08    35.78125     1.444444
#> p__Bacteria_Rank_Firmicutes       5.4868e-06 11705.71875 25826.555556
#> p__Bacteria_Rank_Patescibacteria  8.2322e-06    78.53125   511.111111
#> p__Bacteria_Rank_Spirochaetota    5.6116e-04     0.96875     7.222222
#> p__Bacteria_Rank_Synergistota     1.4714e-02     0.96875     5.555556
#>                                   M.tb_Uninfected          max          min
#> p__Bacteria_Rank_Bacteroidota         9532.258065  9532.258065   420.906250
#> p__Bacteria_Rank_Fusobacteriota       3425.903226  5374.222222   231.531250
#> p__Bacteria_Rank_Campylobacterota       49.483871    88.888889     4.812500
#> p__Bacteria_Rank_Proteobacteria       7755.483871 13637.888889  1883.031250
#> p__Bacteria_Rank_Actinobacteriota     2464.387097 34977.718750  2464.387097
#> k__Unknown                              12.419355    35.781250     1.444444
#> p__Unknown_Rank_Unknown                 12.419355    35.781250     1.444444
#> p__Bacteria_Rank_Firmicutes           25587.935484 25826.555556 11705.718750
#> p__Bacteria_Rank_Patescibacteria        458.322581   511.111111    78.531250
#> p__Bacteria_Rank_Spirochaetota           16.580645    16.580645     0.968750
#> p__Bacteria_Rank_Synergistota             2.612903     5.555556     0.968750
```

```
#>                                  LDAscore           class    Pvalues     color
#> p__Bacteria_Rank_Bacteroidota        3.660 M.tb_Uninfected 4.1243e-10      blue
#> p__Bacteria_Rank_Fusobacteriota      3.410            LTBI 1.3148e-09       red
#> p__Bacteria_Rank_Campylobacterota    1.630            LTBI 2.7886e-09       red
#> p__Bacteria_Rank_Proteobacteria      3.770            LTBI 5.9360e-09       red
#> p__Bacteria_Rank_Actinobacteriota    4.210          Active 1.3276e-08 darkgreen
#> k__Unknown                           1.260          Active 4.8022e-08 darkgreen
#> p__Unknown_Rank_Unknown              1.260          Active 4.8022e-08 darkgreen
#> p__Bacteria_Rank_Firmicutes          3.850            LTBI 3.1353e-06       red
#> p__Bacteria_Rank_Patescibacteria     2.340            LTBI 5.2922e-06       red
#> p__Bacteria_Rank_Spirochaetota       0.945 M.tb_Uninfected 4.0083e-04      blue
#> p__Bacteria_Rank_Synergistota        0.518            LTBI 1.1561e-02       red

# Save the Rank 2 tree plot as a high-resolution image
ggsave(filename = "Results/Figure 8A.jpg",
       plot = tree_plots[["Rank_2_tree_plot"]],
       width = 10, height = 8, dpi = 600)

# Save the Rank 2 bar plot as a high-resolution image
ggsave(filename = "Results/Figure 8B.jpg",
       plot = bar_plots[["Rank_2_bar_plot"]],
       width = 10, height = 8, dpi = 600)
```

Machine Learning

Compare machine learning models

```
MicroRoc <- function(otu = NULL, tax = NULL, map = NULL, tree = NULL,
                     ps = NULL, group_var = NULL, repnum = 5) {

  # Prepare the phyloseq object for analysis
  ps <- ggClusterNet::inputMicro(otu, tax, map, tree, ps, group = group_var)

  # Extract sample data and OTU table from the phyloseq object
  sample_mapping <- as.data.frame(phyloseq::sample_data(ps))

# Ensure the group_var is treated as a factor with explicit levels
sample_mapping[[group_var]] <- factor(sample_mapping[[group_var]])

# Convert the factor levels to numeric: 0 for the first level, 1 for the second
sample_mapping[[group_var]] <- as.numeric(sample_mapping[[group_var]]) - 1

# Check the conversion
 table(sample_mapping[[group_var]])  # Should show counts of 0s and 1s

  otu_table <- as.data.frame(t(ggClusterNet::vegan_otu(ps)))
  colnames(otu_table) <- gsub("-", "_", colnames(otu_table))

  # Prepare the data for modeling
  data_for_modeling <- as.data.frame(t(otu_table))
```

```r
data_for_modeling$group <- factor(sample_mapping[[group_var]])  # Ensure group is a factor
colnames(data_for_modeling) <- paste(colnames(data_for_modeling), sep = "")

# Random Forest, SVM, and GLM models
models <- c("RF", "SVM", "GLM")
auc_scores <- list()
roc_data <- list()
set.seed(100)  # Use the same seed value for reproducibility

for (model in models) {
  auc_values <- numeric(repnum)  # Store AUC for each fold
  tpr_fpr_list <- list()  # Store TPR and FPR for each fold

  folds <- createFolds(y = data_for_modeling$group, k = repnum)

  for (i in 1:repnum) {
    test_fold <- data_for_modeling[folds[[i]], ]
    train_fold <- data_for_modeling[-folds[[i]], ]

    if (model == "RF") {
      rf_model <- randomForest(group ~ ., data = train_fold, importance = TRUE)
      rf_predictions <- predict(rf_model, newdata = test_fold, type = "prob")[, 2]

      roc_result <- roc(test_fold$group, rf_predictions)
      auc_values[i] <- auc(roc_result)
      tpr_fpr_list[[i]] <- data.frame(tpr = roc_result$sensitivities, fpr = 1 - roc_result$specificit

    } else if (model == "SVM") {
      svm_model <- svm(group ~ ., data = train_fold, probability = TRUE)
      svm_predictions <- attr(predict(svm_model, test_fold, probability = TRUE), "probabilities")[, 2

      roc_result <- roc(test_fold$group, svm_predictions)
      auc_values[i] <- auc(roc_result)
      tpr_fpr_list[[i]] <- data.frame(tpr = roc_result$sensitivities, fpr = 1 - roc_result$specificit

    } else if (model == "GLM") {
      glm_model <- glm(group ~ ., family = binomial, data = train_fold)
      glm_predictions <- predict(glm_model, test_fold, type = "response")

      roc_result <- roc(test_fold$group, glm_predictions)
      auc_values[i] <- auc(roc_result)
      tpr_fpr_list[[i]] <- data.frame(tpr = roc_result$sensitivities, fpr = 1 - roc_result$specificit
    }
  }

  # Calculate mean AUC and 95% CI for the model
  mean_auc <- mean(auc_values)
  auc_ci <- quantile(auc_values, probs = c(0.025, 0.975))  # 95% CI using quantiles

  # Combine TPR/FPR data across folds and calculate mean TPR at each FPR
  combined_roc_data <- bind_rows(tpr_fpr_list)
  mean_roc <- combined_roc_data %>%
    group_by(fpr) %>%
```

```
      summarise(mean_tpr = mean(tpr, na.rm = TRUE),
                lower_ci = quantile(tpr, 0.025, na.rm = TRUE),
                upper_ci = quantile(tpr, 0.975, na.rm = TRUE))

    # Store AUC results and ROC curve data
    auc_scores[[model]] <- list(mean = mean_auc, ci = auc_ci)
    roc_data[[model]] <- mean_roc
  }

# Function to create individual ROC plots with CI
plot_roc <- function(roc_data, auc, model_name, color) {
  ggplot(roc_data, aes(x = fpr)) +
    # Shaded region representing the 95% confidence interval
    geom_ribbon(aes(ymin = lower_ci, ymax = upper_ci), fill = color, alpha = 0.2) +
    # Mean ROC curve line
    geom_line(aes(y = mean_tpr), color = color, size = 1) +
    # Labels and title
    labs(x = "False Positive Rate",
         y = "True Positive Rate",
         title = sprintf("Performance of the %s Machine Learning Model", model_name)) +
    # AUC text annotation
    annotate("text", x = 0.75, y = 0.25, label = sprintf("AUC: %.3f (95%% CI: %.3f - %.3f)",
                                                         auc$mean, auc$ci[1], auc$ci[2]), color = color) +
    # Improved minimal theme for better aesthetics
    theme_minimal(base_size = 14) +
    # Ensure that grid lines and axis labels are clear
    theme(
      plot.title = element_text(hjust = 0.5, size = 16, face = "bold"),
      axis.title = element_text(size = 14),
      axis.text = element_text(size = 12),
      panel.grid.minor = element_blank()
    )
}
# Example of how this is used:
# Create separate plots for RF, SVM, and GLM
rf_plot <- plot_roc(roc_data$RF, auc_scores$RF, "Random Forest", "red")
svm_plot <- plot_roc(roc_data$SVM, auc_scores$SVM, "SVM", "blue")
glm_plot <- plot_roc(roc_data$GLM, auc_scores$GLM, "GLM", "black")


return(list(rf_plot = rf_plot, svm_plot = svm_plot, glm_plot = glm_plot, auc_scores = auc_scores))

}
# Example usage
result <- MicroRoc(ps = Genexpert_phyloseq, group_var = "Group")
#> Error in Math.factor(exp0): 'cumsum' not meaningful for factors
print(result$rf_plot)
#> Error: object 'result' not found
print(result$svm_plot)
#> Error: object 'result' not found
print(result$glm_plot)
#> Error: object 'result' not found
print(result$auc_scores)
```

```
#> Error: object 'result' not found
```

```
MicroRoc <- function(otu = NULL, tax = NULL, map = NULL, tree = NULL,
                     ps = NULL, group_var = NULL, repnum = 5) {

  # Prepare the phyloseq object for analysis
  ps <- ggClusterNet::inputMicro(otu, tax, map, tree, ps, group = group_var)

  # Extract sample data and OTU table from the phyloseq object
  sample_mapping <- as.data.frame(phyloseq::sample_data(ps))

  # Ensure the group_var is treated as a factor with explicit levels
  sample_mapping[[group_var]] <- factor(sample_mapping[[group_var]])

  # Convert the factor levels to numeric: 0 for the first level, 1 for the second
  sample_mapping[[group_var]] <- as.numeric(sample_mapping[[group_var]]) - 1

  # Check the conversion
  table(sample_mapping[[group_var]])  # Should show counts of 0s and 1s

  otu_table <- as.data.frame(t(ggClusterNet::vegan_otu(ps)))
  colnames(otu_table) <- gsub("-", "_", colnames(otu_table))

  # Prepare the data for modeling
  data_for_modeling <- as.data.frame(t(otu_table))
  data_for_modeling$group <- factor(sample_mapping[[group_var]])  # Ensure group is a factor

  # Models to evaluate
  models <- c("RF", "SVM", "GLM")
  auc_scores <- list()
  roc_data <- list()

  # Define a common FPR grid
  common_fpr <- seq(0, 1, length.out = 100)

  set.seed(100)  # Use the same seed value for reproducibility

  for (model in models) {
    auc_values <- numeric(repnum)  # Store AUC for each fold
    tpr_list <- matrix(NA, nrow = length(common_fpr), ncol = repnum)  # Store interpolated TPR values

    folds <- createFolds(y = data_for_modeling$group, k = repnum)

    for (i in 1:repnum) {
      test_fold <- data_for_modeling[folds[[i]], ]
      train_fold <- data_for_modeling[-folds[[i]], ]

      if (model == "RF") {
        model_fit <- randomForest(group ~ ., data = train_fold, importance = TRUE)
        predictions <- predict(model_fit, newdata = test_fold, type = "prob")[, 2]

      } else if (model == "SVM") {
        model_fit <- svm(group ~ ., data = train_fold, probability = TRUE)
```

```
      predictions <- attr(predict(model_fit, test_fold, probability = TRUE), "probabilities")[, 2]

    } else if (model == "GLM") {
      model_fit <- glm(group ~ ., family = binomial, data = train_fold)
      predictions <- predict(model_fit, test_fold, type = "response")
    }

    # Compute ROC and interpolate TPR at common FPR
    # Ensure test_fold$group is numeric
roc_result <- roc(as.numeric(as.character(test_fold$group)), predictions)
    interpolated_tpr <- approx(x = roc_result$specificities,
                               y = roc_result$sensitivities,
                               xout = common_fpr,
                               method = "linear",
                               ties = "ordered")$y
    tpr_list[, i] <- interpolated_tpr
    auc_values[i] <- auc(roc_result)
  }

  # Calculate mean and CI for TPR at each FPR
  mean_tpr <- rowMeans(tpr_list, na.rm = TRUE)
  lower_ci <- apply(tpr_list, 1, quantile, probs = 0.025, na.rm = TRUE)
  upper_ci <- apply(tpr_list, 1, quantile, probs = 0.975, na.rm = TRUE)

  # Store AUC results and ROC curve data
  auc_scores[[model]] <- list(mean = mean(auc_values), ci = quantile(auc_values, c(0.025, 0.975)))
  roc_data[[model]] <- data.frame(fpr = common_fpr, mean_tpr = mean_tpr, lower_ci = lower_ci, upper_ci
}

# Function to create individual ROC plots with CI
plot_roc <- function(roc_data, auc, model_name, color) {
  ggplot(roc_data, aes(x = fpr)) +
    geom_ribbon(aes(ymin = lower_ci, ymax = upper_ci), fill = color, alpha = 0.2) +
    geom_line(aes(y = mean_tpr), color = color, size = 1) +
    labs(x = "False Positive Rate",
         y = "True Positive Rate",
         title = sprintf("Performance of the %s Model", model_name)) +
    annotate("text", x = 0.75, y = 0.25,
             label = sprintf("AUC: %.3f (95%% CI: %.3f - %.3f)", auc$mean, auc$ci[1], auc$ci[2]), colo
    theme_minimal(base_size = 14)
}

# Create plots for RF, SVM, and GLM
rf_plot <- plot_roc(roc_data$RF, auc_scores$RF, "Random Forest", "red")
svm_plot <- plot_roc(roc_data$SVM, auc_scores$SVM, "SVM", "blue")
glm_plot <- plot_roc(roc_data$GLM, auc_scores$GLM, "GLM", "black")

# Save each plot
ggsave(filename = "Results/Figure 9A.jpg", plot = rf_plot, width = 8, height = 6, dpi = 600)
ggsave(filename = "Results/Figure 9B.jpg", plot = svm_plot, width = 8, height = 6, dpi = 600)
ggsave(filename = "Results/Figure 9C.jpg", plot = glm_plot, width = 8, height = 6, dpi = 600)

return(list(rf_plot = rf_plot, svm_plot = svm_plot, glm_plot = glm_plot, auc_scores = auc_scores))
```

```
}
# Example usage
result <- MicroRoc(ps = Genexpert_phyloseq, group_var = "Group")
#> Error in approx(x = roc_result$specificities, y = roc_result$sensitivities, : need at least two non-
print(result$rf_plot)
#> Error: object 'result' not found
print(result$svm_plot)
#> Error: object 'result' not found
print(result$glm_plot)
#> Error: object 'result' not found
print(result$auc_scores)
#> Error: object 'result' not found
```

Random Forest model

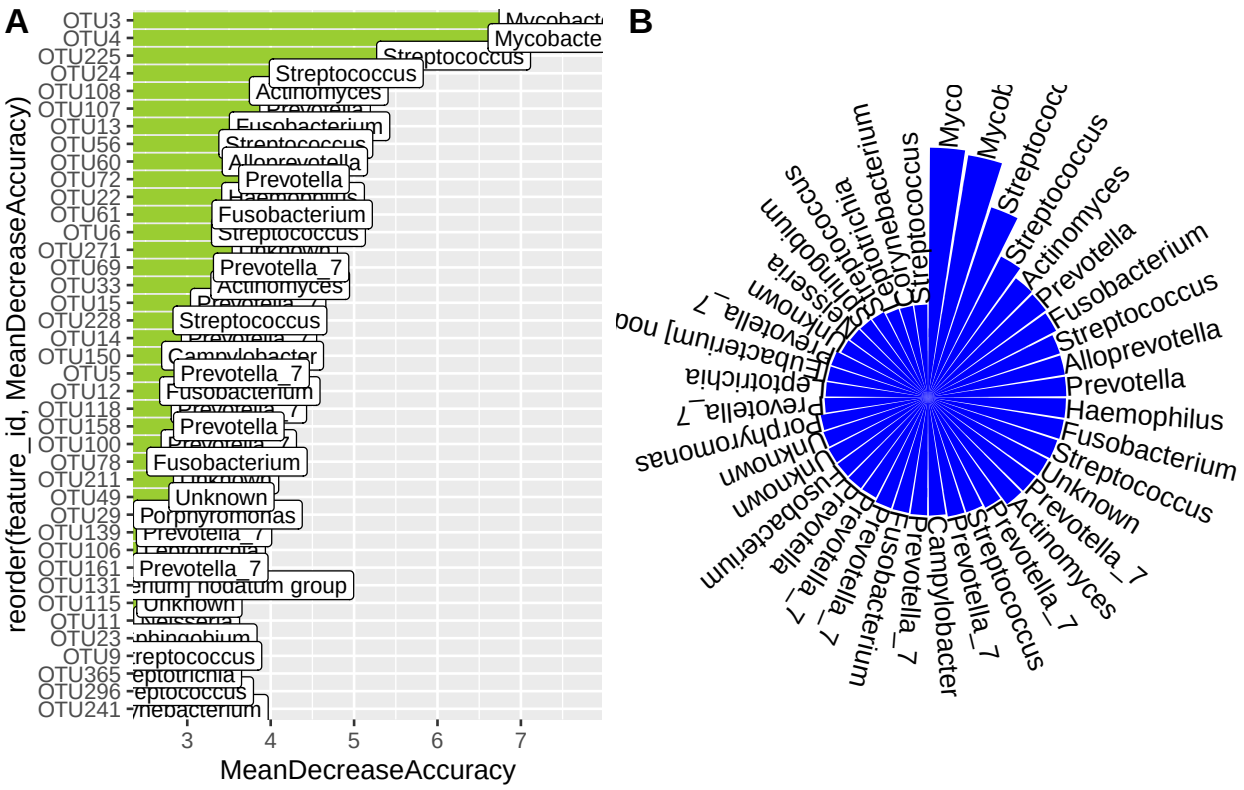
```
# Function to estimate model accuracy and generate a confusion matrix table
estimate_accuracy <- function(rf_model) {
  # Extract confusion matrix from the Random Forest model
  confusion_matrix <- as.data.frame(rf_model$confusion)

  # Add a new column to calculate the class error
  confusion_matrix$class_error <- round(confusion_matrix$class.error, 3)
  confusion_matrix$Group <- row.names(confusion_matrix)

  # Reorder columns for better readability
  confusion_matrix <- dplyr::select(confusion_matrix, Group, everything())

  # Calculate the overall accuracy rate of the model
  accuracy_rate <- paste(round(100 - tail(rf_model$err.rate[, 1], 1) * 100, 2), "%", sep = "")
  accuracy_rate_df <- data.frame(ID = "Model Accuracy Rate", Accuracy_Rate = accuracy_rate)
  colnames(accuracy_rate_df) <- c("Random Forest", "Accuracy Rate")

  # Create tables for the accuracy rate and confusion matrix
  accuracy_table <- ggpubr::ggtexttable(accuracy_rate_df, rows = NULL)
  confusion_table <- ggpubr::ggtexttable(confusion_matrix, rows = NULL)

  # Combine both tables into one display
  combined_tables <- accuracy_table / confusion_table

  # Return the combined tables
  return(combined_tables)
}

# Function to extract top important features (bacterial markers) based on Random Forest model
extract_top_features <- function(rf_model, ps, top_features = 20) {
  # Extract feature importance metrics from the Random Forest model
  feature_importance <- as.data.frame(round(randomForest::importance(rf_model), 2))

  # Assign feature IDs for better readability
  feature_importance$feature_id <- row.names(feature_importance)
  row.names(feature_importance) <- gsub("feature", "", row.names(feature_importance))
```

```
  feature_importance$feature_id <- gsub("feature", "", feature_importance$feature_id)

  # Order features by their importance (MeanDecreaseAccuracy) and select top features
  ordered_features <- dplyr::arrange(feature_importance, desc(MeanDecreaseAccuracy))
  top_features_df <- head(ordered_features, n = top_features)

  # Extract taxonomy information corresponding to the top features
  taxonomy_df <- as.data.frame(ggClusterNet::vegan_tax(ps))
  relevant_taxonomy <- taxonomy_df[rownames(top_features_df), ]

  # Merge top features with their corresponding taxonomy information
  top_features_df <- merge(top_features_df, relevant_taxonomy, by = "row.names", all = FALSE)
  row.names(top_features_df) <- top_features_df$Row.names
  top_features_df$Row.names <- NULL

  # Return the dataframe of top features along with their taxonomy
  return(top_features_df)
}

# Function to plot the importance of features and create a polar bar plot
plot_feature_importance <- function(top_features_df) {
  # Plot 1: Importance of features based on MeanDecreaseAccuracy from Random Forest
  importance_plot <- ggplot(top_features_df, aes(x = MeanDecreaseAccuracy, y = reorder(feature_id, Mean
    geom_point(size = 6, pch = 21, fill = "#9ACD32", color = "#9ACD32") +
    geom_segment(aes(yend = feature_id), xend = 0, size = 3, color = "#9ACD32") +
    geom_label(aes(x = MeanDecreaseAccuracy * 1.1, label = Genus), size = 3)

  # Prepare data for the polar plot by arranging and indexing the top features
  top_features_df <- dplyr::arrange(top_features_df, desc(MeanDecreaseAccuracy))
  top_features_df$index <- paste(1:length(top_features_df$feature_id))
  label_angle <- 90 - 360 * (as.numeric(top_features_df$index) - 0.5) / length(top_features_df$feature_
  top_features_df$feature_id <- factor(top_features_df$feature_id, levels = top_features_df$feature_id)

  # Plot 2: Polar bar plot of feature importance
  polar_plot <- top_features_df %>%
    ggplot(aes(x = factor(feature_id), y = MeanDecreaseAccuracy, label = Genus)) +
    geom_bar(stat = 'identity', position = 'dodge', fill = "blue") +
    geom_text(hjust = 0, angle = label_angle, alpha = 1) +
    coord_polar() +
    theme_void()

  # Return the plots
  return(list(importance_plot = importance_plot, polar_plot = polar_plot))
}

# Function to perform recursive feature elimination cross-validation (RFCV) with statistics
run_recursive_feature_cv <- function(otu = NULL, tax = NULL, map = NULL, tree = NULL,
                                     ps = NULL, group = "Group", optimal_features = 20, num_folds = 5) {

  # Preprocess the phyloseq object and scale the microbial data
  ps <- ggClusterNet::inputMicro(otu, tax, map, tree, ps, group = group)

  # Prepare the OTU table and sample metadata
```

```r
  otu_table <- as.data.frame(ggClusterNet::vegan_otu(ps))
  sample_metadata <- as.data.frame(phyloseq::sample_data(ps))

  # Set classification labels and clean up column names
  otu_table$group <- factor(sample_metadata$Group)
  colnames(otu_table) <- gsub("-", "_", colnames(otu_table))

  # Define feature data by removing the group column
  num_features <- ncol(otu_table) - 1
  feature_data <- otu_table[1:num_features]

  # Initial RFCV with a set seed
  set.seed(315)
  rfcv_result <- rfcv(feature_data, otu_table$group, cv.fold = num_folds, scale = "log", step = 0.9)

  # Store cross-validation error data for each fold
  error_cv_df <- data.frame(num_features = rfcv_result$n.var, error_rate_1 = rfcv_result$error.cv)

  # Repeat RFCV with different seeds to ensure stability
  for (seed in 316:(315 + num_folds - 1)) {
    set.seed(seed)
    rfcv_result <- rfcv(feature_data, otu_table$group, cv.fold = num_folds, scale = "log", step = 0.9)
    error_cv_df <- cbind(error_cv_df, rfcv_result$error.cv)
  }

  # Calculate mean, standard deviation, and confidence intervals for error rates
  error_cv_df <- error_cv_df[, -1]  # Remove the num_features column
  colnames(error_cv_df) <- paste('error_rate', 1:num_folds, sep = '_')
  mean_error <- rowMeans(error_cv_df)
  std_error <- apply(error_cv_df, 1, sd)

  # Calculate 95% confidence intervals (assuming normal distribution)
  ci_low <- mean_error - 1.96 * (std_error / sqrt(num_folds))
  ci_high <- mean_error + 1.96 * (std_error / sqrt(num_folds))

  # Prepare summary data for plotting and reporting
  rfcv_summary <- data.frame(
    num_features = rfcv_result$n.var,
    mean_error = mean_error,
    std_error = std_error,
    ci_low = ci_low,
    ci_high = ci_high
  )

  # Transform the data for plotting
  plot_data <- tidyr::gather(rfcv_summary, key = "metric", value = "value", -num_features)

 # Generate a plot showing the RFCV results with error bars
rfcv_plot <- ggplot() +
  geom_line(data = plot_data, aes(x = num_features, y = value, group = metric, color = metric), linetyp
  geom_ribbon(aes(x = rfcv_summary$num_features, ymin = rfcv_summary$ci_low, ymax = rfcv_summary$ci_hig
  geom_line(aes(x = rfcv_summary$num_features, y = rfcv_summary$mean_error), colour = 'black') +
  coord_trans(x = "log2") +
```

```
  scale_x_continuous(breaks = c(1, 2, 5, 10, 20, 30, 50, 100, 200)) +
  labs(title = paste('Cross validation Training set (n =', nrow(otu_table), ')', sep = ''),
       x = 'Number of features',
       y = 'Cross-validation error rate') +
  theme(
    plot.title = element_text(size = 16, face = "bold", hjust = 0.5),  # Customize title size and posit
    axis.title.x = element_text(size = 14),  # Customize x-axis title size
    axis.title.y = element_text(size = 14)   # Customize y-axis title size
  ) +
  annotate("text", x = optimal_features, y = max(rfcv_summary$mean_error),
           label = paste("optimal = ", optimal_features, sep = ""))

# Return the plot and summary data
return(list(plot = rfcv_plot, summary_data = rfcv_summary))
}

# Main function to run the Random Forest analysis and generate reports and plots
micro_rf_analysis <- function(otu = NULL, tax = NULL, map = NULL, tree = NULL,
                              ps = NULL, group = "Group", top_features = 20, perform_rfcv = FALSE,
                              rfcv_folds = 5, min_ylim = -1, max_ylim = 5) {

  # Preprocess the phyloseq object and scale the microbial data
  ps <- ggClusterNet::inputMicro(otu, tax, map, tree, ps, group = group) %>% ggClusterNet::scale_micro()
  sample_data_df <- as.data.frame(phyloseq::sample_data(ps))

  # Prepare OTU table with scaled data and set up Random Forest model
  mapping_df <- as.data.frame(phyloseq::sample_data(ps))
  otu_table <- as.data.frame((ggClusterNet::vegan_otu(ps)))
  colnames(otu_table) <- paste("feature", colnames(otu_table), sep = "")
  otu_table$group <- factor(mapping_df$Group)

  # Fit the Random Forest model
  rf_model <- randomForest::randomForest(group ~ ., data = otu_table, importance = TRUE, proximity = TRU
  print(rf_model)

  # Estimate the accuracy of the model and generate the confusion matrix
  accuracy_table <- estimate_accuracy(rf_model)

  # Extract the top important features (bacterial markers)
  top_features_df <- extract_top_features(rf_model, ps, top_features)

  # Generate plots for feature importance
  plots <- plot_feature_importance(top_features_df)

  # Perform RFCV if requested
  if (perform_rfcv) {
    rfcv_result <- run_recursive_feature_cv(otu = NULL, tax = NULL, map = NULL, tree = NULL,
                                            ps = ps, group = group, optimal_features = top_features,
                                            num_folds = rfcv_folds)
    rfcv_plot <- rfcv_result[[1]]
    rfcv_table <- rfcv_result[[2]]
  } else {
    rfcv_plot <- NULL
```

```
    rfcv_table <- NULL
  }

  # Return the results: importance plot, polar plot, RFCV plot, RFCV table, top features, accuracy tabl
  return(list(plots$importance_plot, plots$polar_plot, rfcv_plot, rfcv_table, top_features_df, accuracy
}

# Run the Random Forest analysis with specified parameters
result <- micro_rf_analysis(ps = MtbInfectionStatus_phyloseq,
                            group = "Group",
                            top_features = 40, perform_rfcv = TRUE, rfcv_folds = 5,
                            min_ylim = -1, max_ylim = 5)
#>
#> Call:
#>  randomForest(formula = group ~ ., data = otu_table, importance = TRUE,      proximity = TRUE)
#>                Type of random forest: classification
#>                      Number of trees: 500
#> No. of variables tried at each split: 22
#>
#>         OOB estimate of  error rate: 19.44%
#> Confusion matrix:
#>                 Active LTBI M.tb_Uninfected class.error
#> Active              30    0               2  0.06250000
#> LTBI                 0    0               9  1.00000000
#> M.tb_Uninfected      2    1              28  0.09677419

# Access the generated plots and tables
plot1 <- result[[1]]  # Importance plot
plot2 <- result[[2]]  # Polar plot
plot3 <- result[[3]]  # RFCV plot (if perform_rfcv = TRUE)

combined_plot <- cowplot::plot_grid(plot1, plot2, labels = c("A", "B"))
# Add a title to the combined plot
combined_plot <- cowplot::ggdraw() +
  cowplot::draw_plot(combined_plot, 0, 0, 1, 0.9) +  # Adjust plot position and size
  cowplot::draw_label("Combined Feature Importance and Polar Plot",
                      x = 0.5, y = 0.95, hjust = 0.5, size = 16)

# Print the combined plot with title
print(combined_plot)
```




```
# Save the plots as high-resolution images
ggsave(filename = "Results/Figure 9D.jpg", plot = combined_plot, width = 10, height = 8, dpi = 600)
ggsave(filename = "Results/Figure 9E.jpg", plot = plot3, width = 10, height = 8, dpi = 600)
```