I-Cultiver, Inc.
Agriculture • Food • Health • Education

VESTA Dramatically Shifts Microbial Communities In Carrots

MICROBIAL COMMUNITIES IN CARROT FIELDS

Carrot Root Communities Are the Most Affected by VESTA Treatment

June 2016

Prepared by:
I-Cultiver, Inc.

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Acknowledgements:

- **VESTA® (SOBEC Corporation, Fowler CA)** is a commercially successful biological product, approved for organic agriculture, and certified pathogen free.
- **I-Cultiver, Inc.** is a technology transfer company, a consortium of interdisciplinary agricultural & food science experts in collaboration to translate fundamental science into applied science. This study was performed through Collaborations with Second Genome, Inc. and CRADA with USDA:
  - Dr. Devin Coleman-Derr, Collaborator & Advisor, I-Cultiver, Inc.
    - PI / Adjunct Assistant Professor, Plant Gene Expression Center, USDA-ARS / UC Berkeley
  - Dr. Rajnish Khanna, Founder, I-Cultiver, Inc.

EXECUTIVE SUMMARY

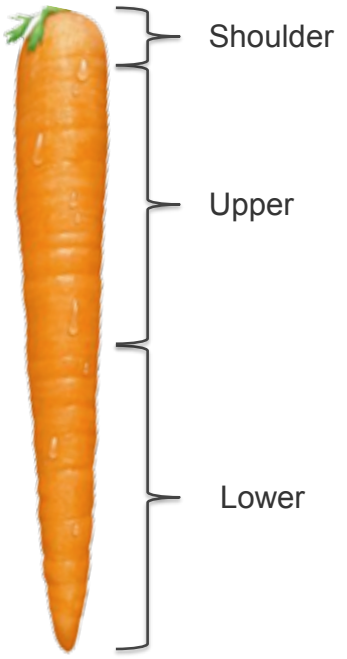
VESTA is a fermented microbial product extensively used in agriculture for disease suppression and growth promotion in multiple commercially valuable crops. In previous studies we have shown that VESTA shifts microbial communities in root endosphere. These shifts include organisms that are not represented in VESTA itself. Our data suggest that the affect of VESTA is modulated by host species, field conditions, or some combination of the two. VESTA organisms likely stimulate the beneficial microbes present in the environment by influencing the metabolic composition and facilitating plant mediated selection of naturally abundant organisms.

VESTA was applied on commercially grown Carrots. This study was conducted to monitor the effect of VESTA application over time from planting to harvest. Three time points were analyzed for expected shifts in Soil, Rhizosphere, and Root Endosphere Microbiota.

This is one of a series of similar studies undertaken. In previous studies we found that VESTA treatment correlated with reduction in Lettuce disease and suppression of the causative pathogen in the soil; in Strawberries, VESTA significantly shifted root microbial communities accompanied with increased Strawberry growth and development. In the present study, VESTA also caused significant community shifts in the Carrot roots. We did not find large, significant overlap in the overrepresented lineages in VESTA treated roots of Carrot and VESTA treated roots of Strawberry, as compared to their controls. The current analysis suggests that the affect of VESTA is largely dependent on the host species, field condition, or some combination of the two. This is consistent with the Strawberry results in which the major shifts in VESTA treated samples were not shifts in organisms that represented a substantial portion of VESTA.

As reported here, VESTA treatment led to enrichment of families and genera, some of which are known to have photosynthetic capacities, nitrogen-fixing abilities, and organisms that prey upon pathogens. Amongst the families and genera suppressed by VESTA treatment were known pathogens and organisms that are nutrient hogs or are known to form kinships with leguminous plant species, and therefor could be deselected by carrots in favor of naturally available beneficial organisms.

CARROT SAMPLES ANALYZED



Samples Analyzed

- **Soil samples from 1st, 2nd, 3rd time points**
- **Rhizosphere samples from 2nd and 3rd time points**
- **Two root samples, one VESTA and one control, from 3rd time point.**
  - **For each root sample, there are three different tissue types:**
    Shoulder (S), Upper (U), and Lower (L)

Based on previous research, we hypothesize that endophytes spatially differ in carrot roots. Shoulders are found to have higher endophyte concentrations compared with other parts of the root (Surette *et al*., 2003. Plant and Soil 253: 381–390).

KEY FINDINGS

- ☑ Microbial community composition was different within sample types (soil, rhizosphere, and root endosphere). The Community composition was significantly influenced by VESTA treatment.

- ☑ VESTA increased species richness in rhizosphere and root endosphere, but not in soil (Note: the carrot varieties being compared in treated and Control samples were different).

- ☑ The greatest difference in OTUs in response to VESTA was in the root endosphere.

- ☑ There were no significant differences observed at the phylum level, but at the family and genera level, suggesting that VESTA caused strain and species substitutions.

- ☑ **Families Enriched in Rhizosphere included: Rhodospirillaceae,** anaerobic photosynthetic bacteria with nitrogen-fixing abilities; **Flexibacteriaceae,** with abilities to oxidize NADH and succinate.

- ☑ **Genera Enriched in Rhizosphere included: Steroidobacter,** capabilities to degrade carrot phytosterols, possibly creating favorable environment for other beneficial microbes; **Nostocaceae,** photosynthetic and nitrogen-fixing capabilities; **Pseudomonas,** metabolic diversity.

- ☑ **Families Enriched in Root Endosphere included: Flexibacteraceae,** (see above).
- ☑ **Families Suppressed in Root Endosphere included: Sphingomonadaceae,** members of this family cause disease in lettuce. We previously reported that VESTA suppresses this family in lettuce fields. These results are consistent with our previous findings. It is not known wether this family is pathogenic to carrots.

- ☑ **Genera Enriched in Root Endosphere included: Myxococcales,** members have been shown to glide over and prey upon *E. coli* colonies (https://www.youtube.com/watch?v=tstc6doiNCU).
- ☑ **Genera Suppressed in Root Endosphere included: BurkholderialesOR,** known pathogenic species; **Bradyrhizobium,** nitrogen-fixers for leguminous plant species, and since carrots are not leguminous, these may be deselected in presence of other favorable nitrogen-fixers (see above); **CaulobacteraceaeFA,** consume nutrients like vitamins and amino acids with no known plant benefit, therefore could be deselected in presence of favorable microbes.

RESULTS

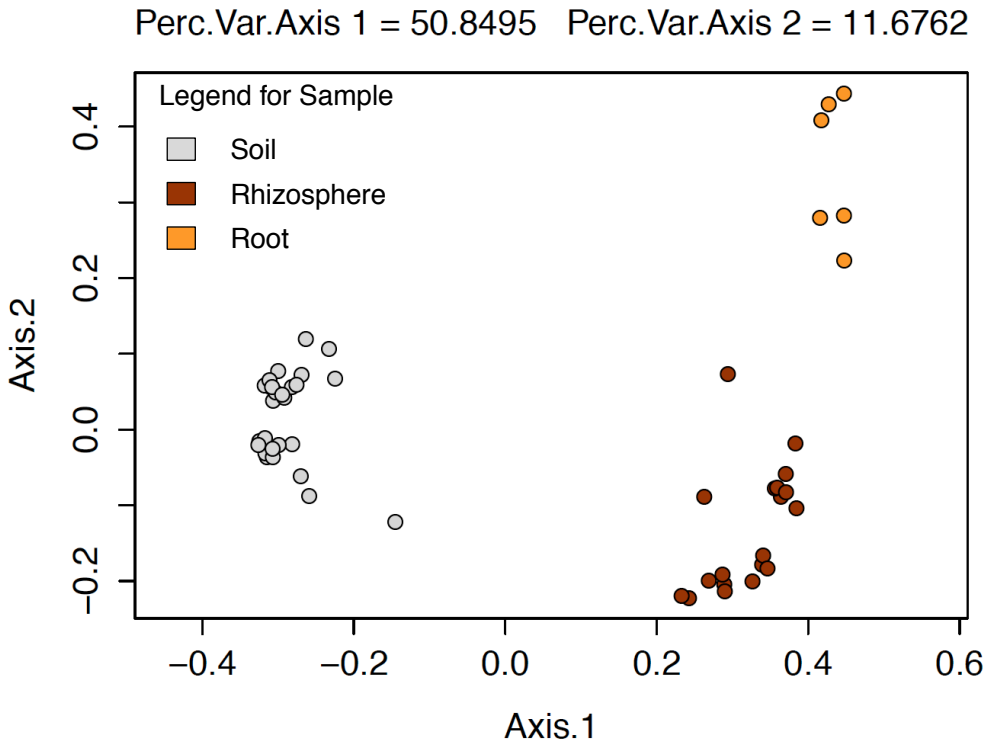


Figure 1. Principal Coordinate Analysis (PCoA). Microbial community composition is determined first by sample type. Soil, Rhizosphere, and Root samples had significantly different community composition.

Anosim Stats:

Sample	0.999	p<0.001
Treatment	0.183	p<0.001
Timepoint	0.016	p=0.267
Replicate	-0.064	p=1.000

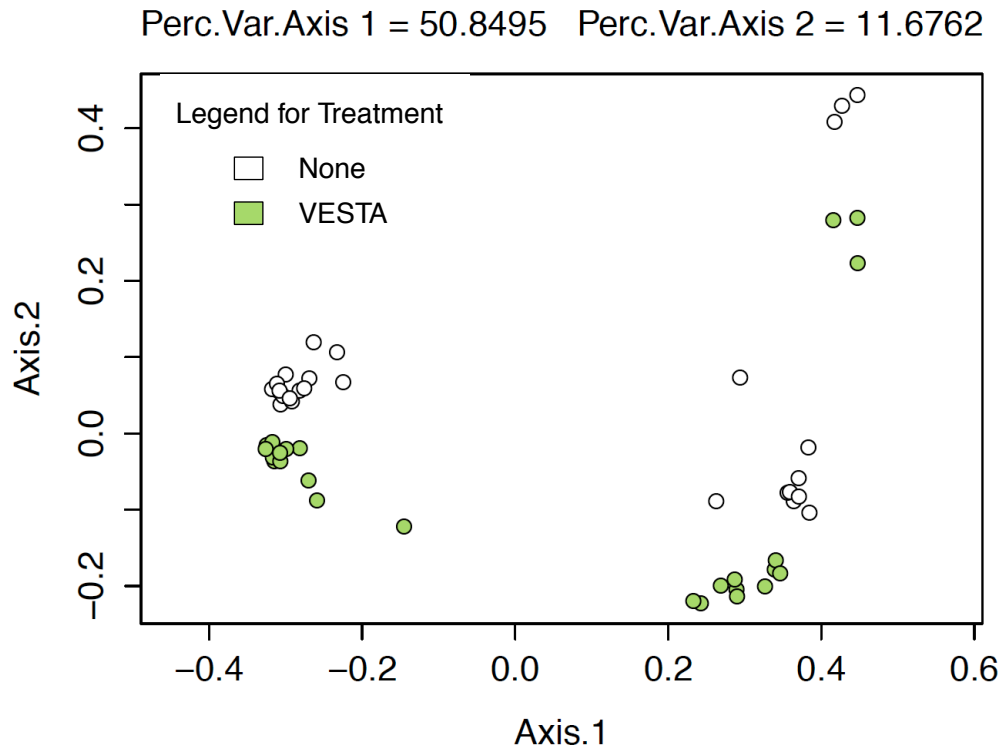


Figure 2. Principal Coordinate Analysis (PCoA). Microbial community composition is determined second by VESTA treatment. The control (None) samples were significantly different from treated (VESTA) samples.

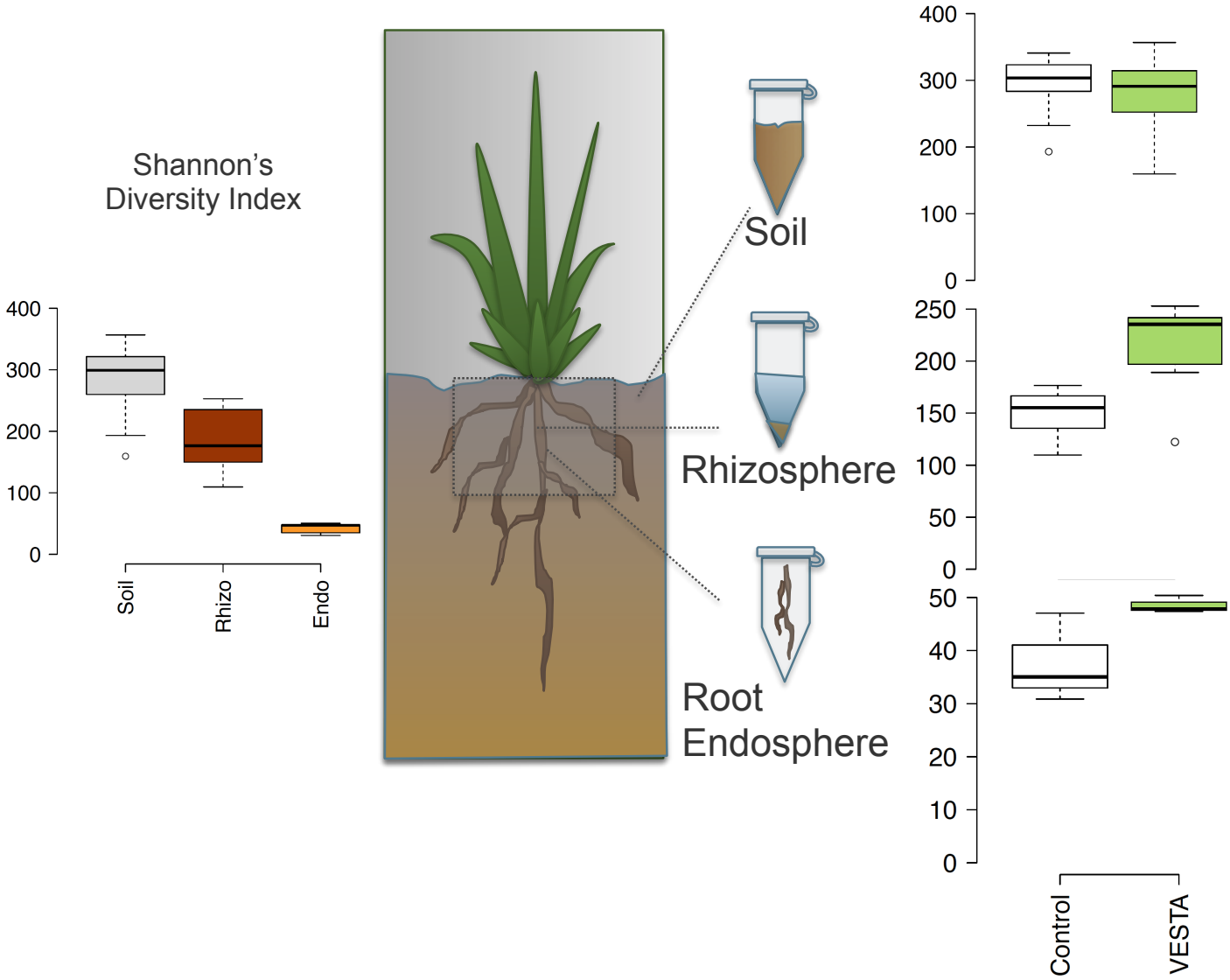


Figure 3. Alpha Diversity: Total community diversity increased in response to VESTA, but only in endosphere and rhizosphere.

- VESTA treatment increased microbial community diversity in rhizosphere and root endosphere.
- In a previous study with strawberries, community diversity was reduced in soil and rhizosphere, and increased in strawberry root endosphere in response to VESTA.
- The increase in carrot community diversity in both Rhizosphere and Endosphere is a new observation and could be specific to carrots and other crops to be determined.
- Carrot roots are the nutrient storage organs of the plant. It is possible that VESTA influences carrot rhizosphere differently than strawberry rhizosphere, where the nutrients are largely stored above ground in the strawberry fruit.
- Community diversity increased in carrot root endosphere, which is similar to the observation with strawberry root endosphere.

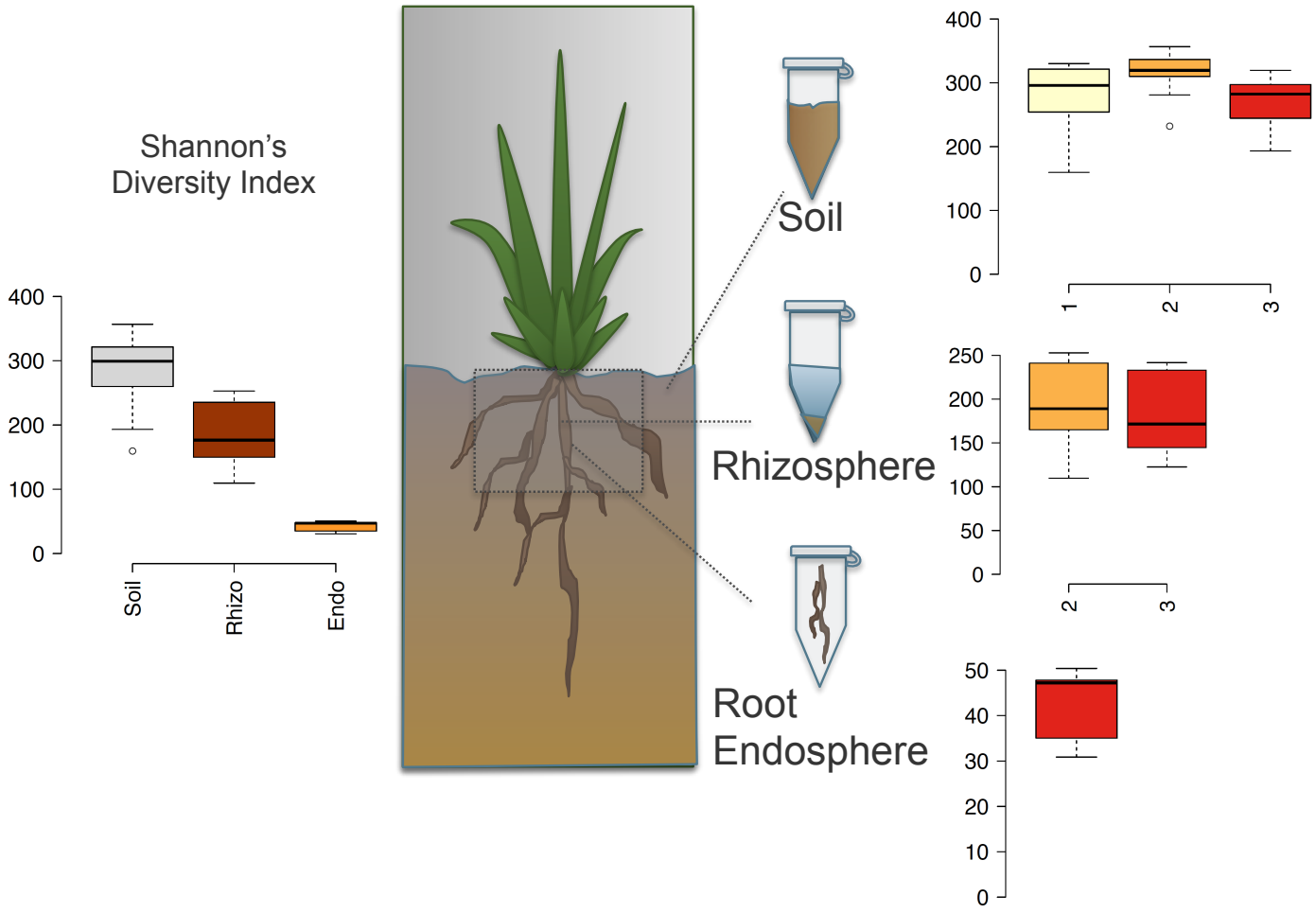


Figure 4. Alpha Diversity: There is no significant difference between time points.

- As seen in the Shannon's Diversity Index plotted on the left, as expected, the overall microbial diversity was highest in soil samples and relatively lower in root endosphere.
- There was no significant difference observed in diversity over the time points tested.
- In strawberries, we previously reported increase in microbial diversity over time in root endosphere. Additional analysis is needed of carrot root endosphere time points to determine if the diversity increases in carrot root endoshere over time.

Figures 3 and 4 show that species richness in soil and carrot rhizosphere was not influenced by time point, but there was an increase in total community diversity in rhizosphere and endosphere in response to VESTA treatment.

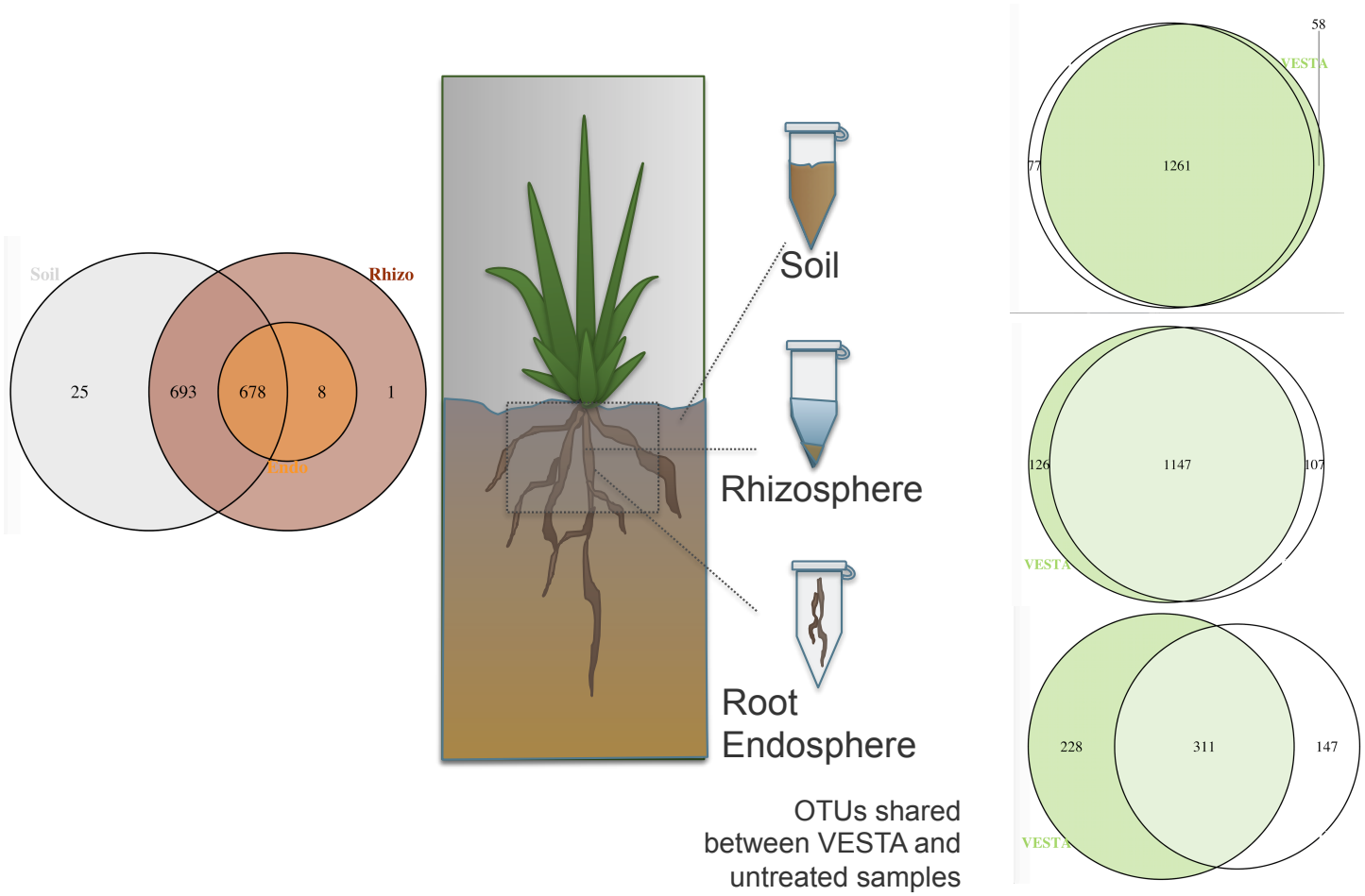


Figure 5. Shared OTUs: The percentage of differentially present OTUs between VESTA and Control increases in roots.

- VESTA treated samples (green circles) shared majority of OTUs with Control (clear circles) samples from soil and rhizosphere.
- In root endosphere, VESTA treated samples had 224 new OTUs that were not found in Control roots, and there were 147 OTUs found in Control samples that were absent in VESTA treated roots.
- These data show that the greatest difference in OTUs was in the root endosphere.

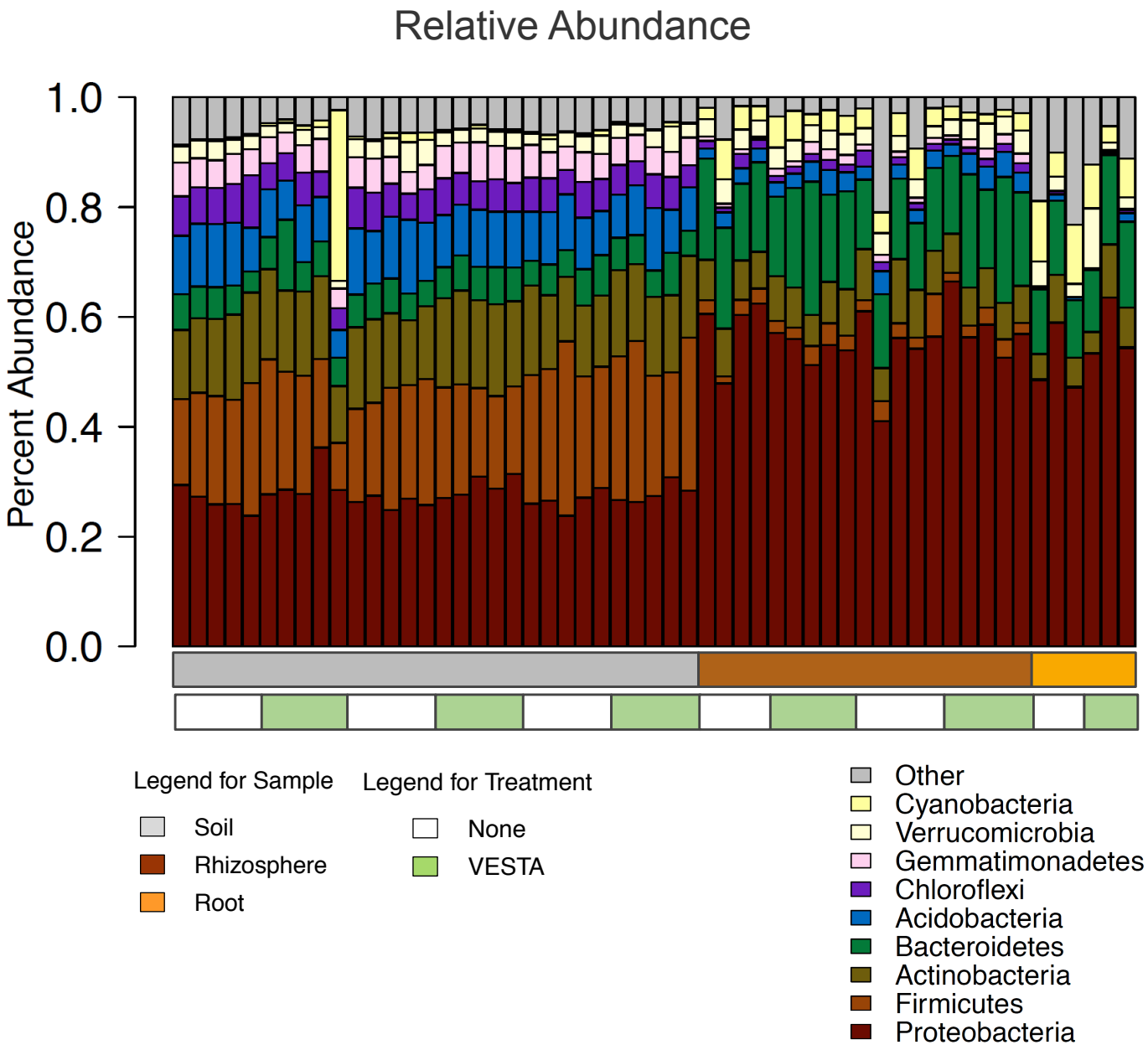


Figure 6. Phylum level relative abundances do not exhibit affect of VESTA treatment on community structure.

- The Control and VESTA treated samples exhibited similar community structures at the Phylum level.

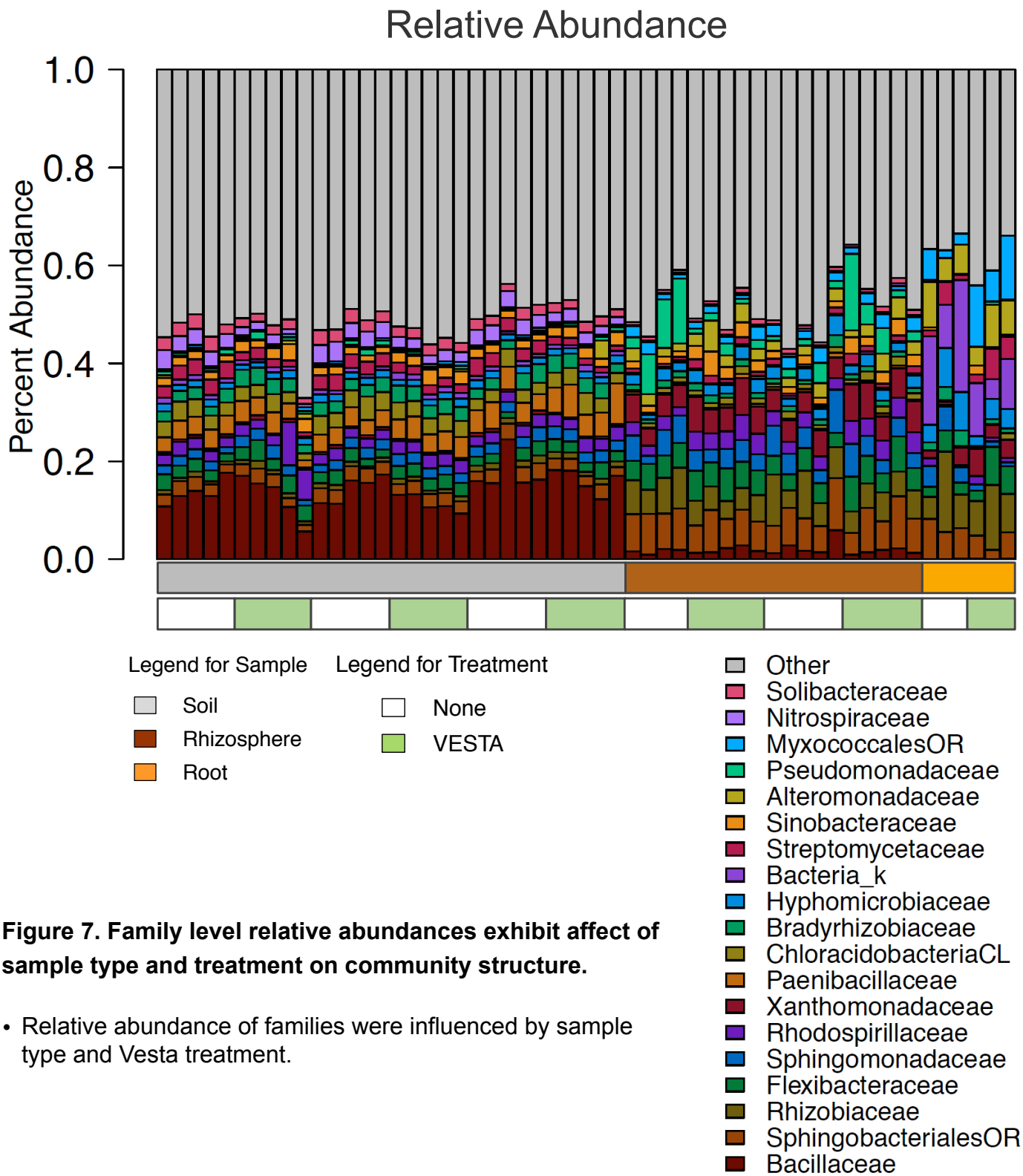
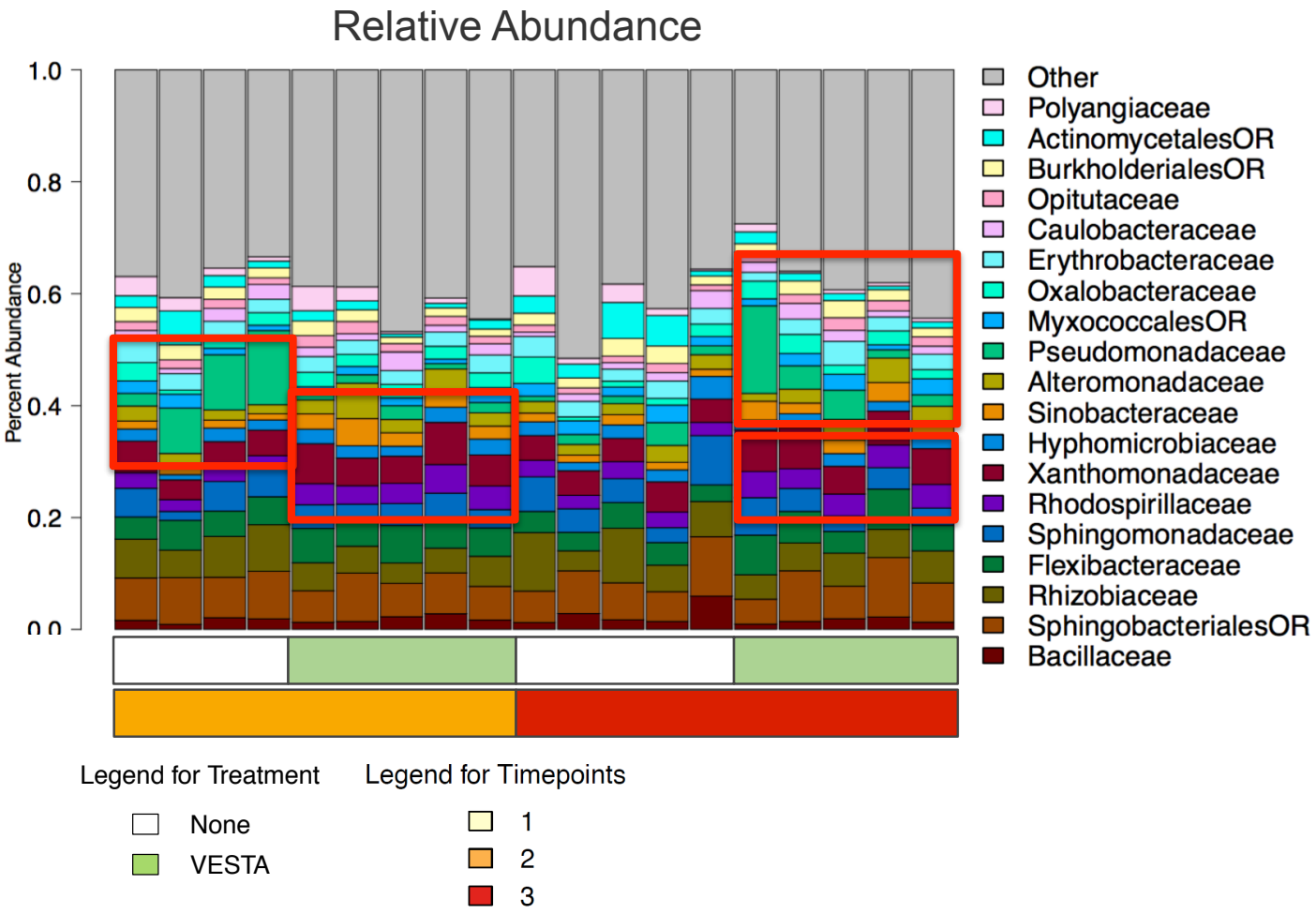


Figure 7. Family level relative abundances exhibit affect of sample type and treatment on community structure.

- Relative abundance of families were influenced by sample type and Vesta treatment.



Timepoint	Enriched in VESTA	Enriched in Control
2	Rhodospirillaceae, Sinobacteraceae	Pseudomonadaceae
3	Rhodospirillaceae, Flexibacteraceae, Pseudomonadaceae	

Figure 8. Families Enriched in the Rhizosphere of VESTA vs. Control Samples

- VESTA treated rhizosphere samples had higher relative abundance of **Rhodospirillaceae** at time points 2 and 3.
- **Rhodospirillaceae** are purple non-sulphur bacteria that belong to the Phylum Proteobacteria. It is a family of anaerobic photosynthetic bacteria, which use organic compounds as substrates for photosynthesis.
- Majority of the **Rhodospirillaceae** species tested had nitrogen-fixing abilities with N_2 as the only nitrogen source under anaerobic photosynthetic conditions (Madigan et al., 1984. J. Bacteriol 73-78).
- Another family enriched in VESTA treated rhizosphere was **Flexibacteriaceae.** Members of this family have been extensively studied for cytochrome o activity, which catalyzes the oxidation of reduced nicotinamide adenine dinucleotide (NADH) and succinate (Dietrich and Biggins 1971. J. Bacteriol 1083-1089).

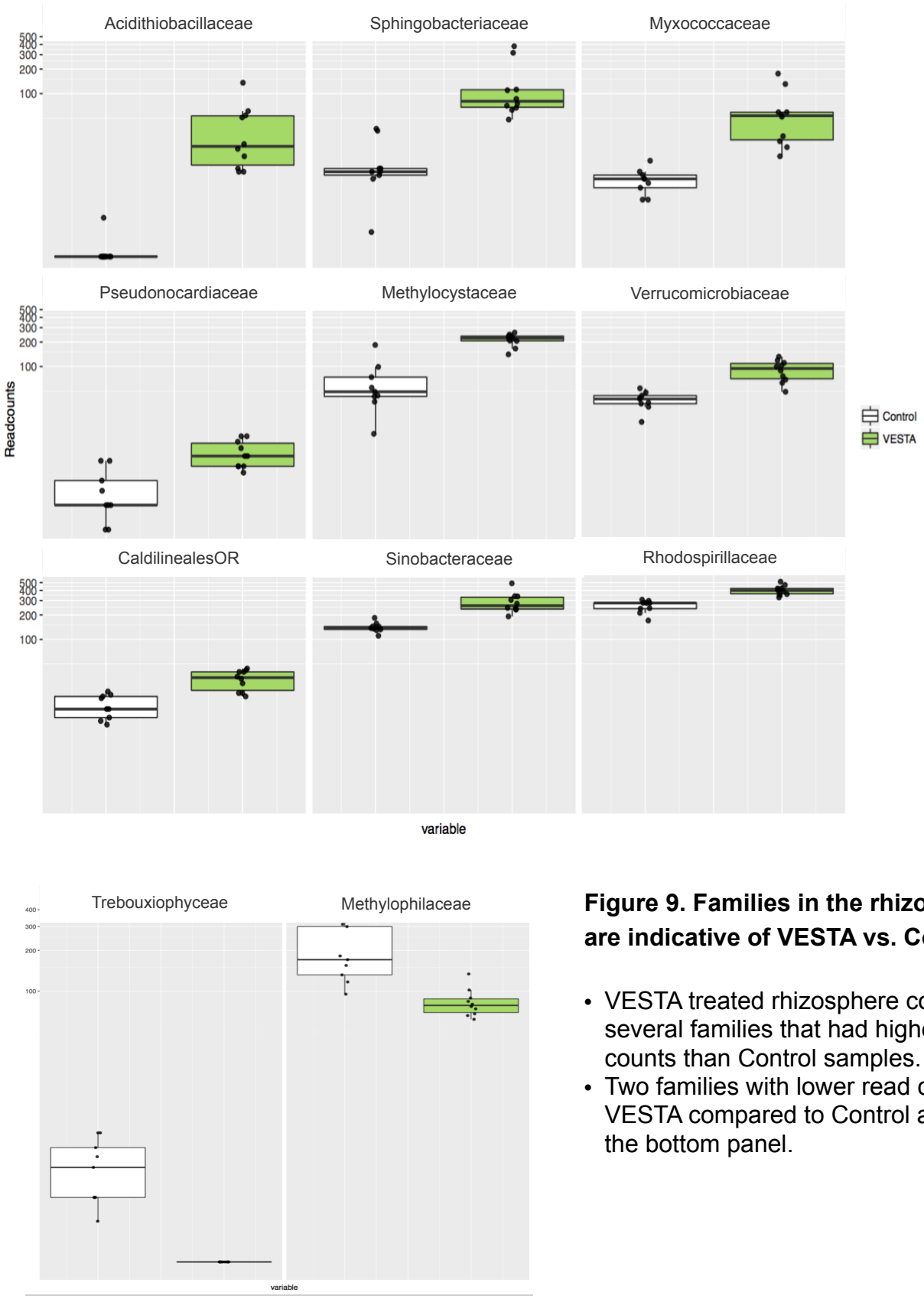
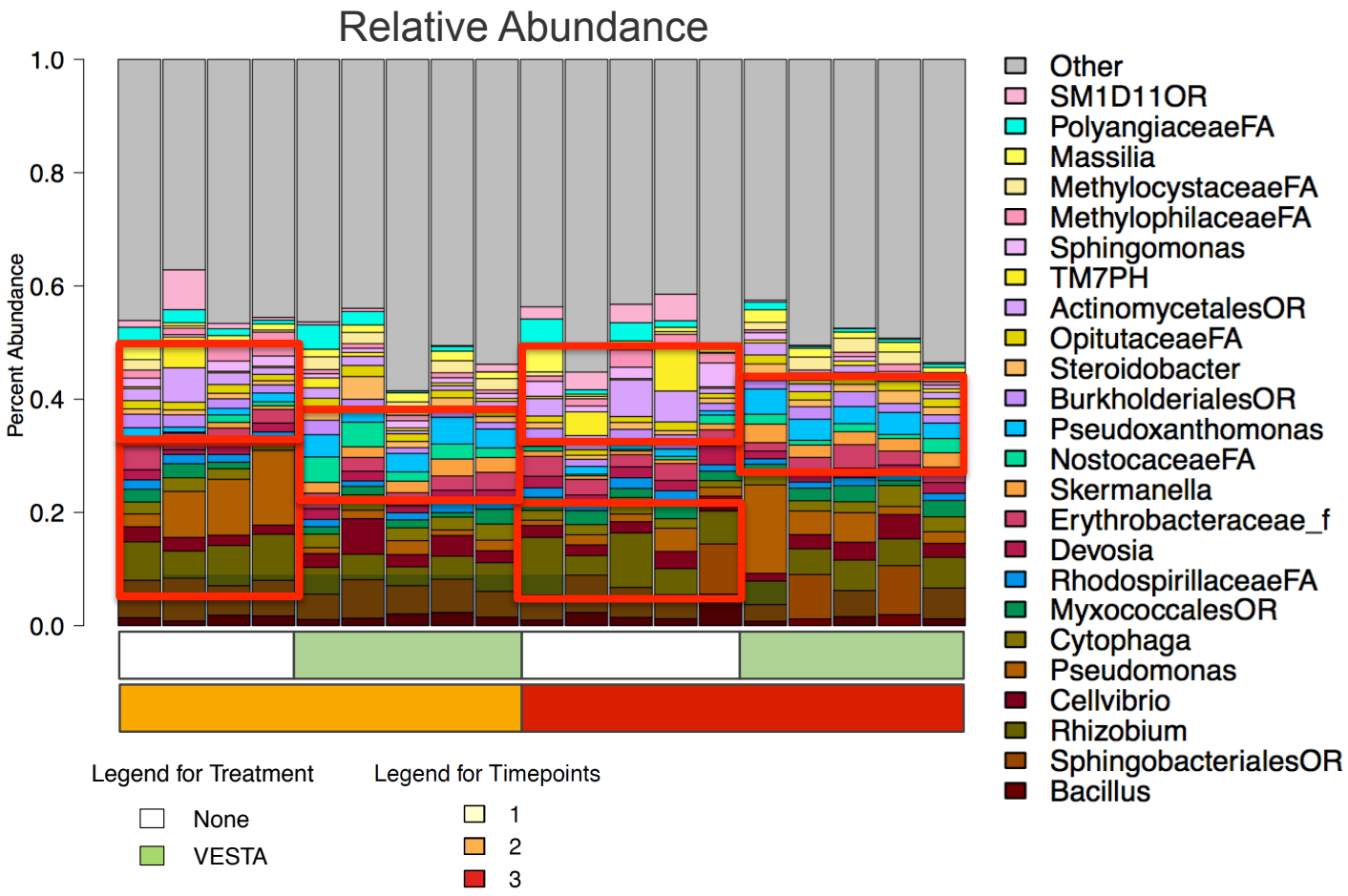


Figure 9. Families in the rhizosphere that are indicative of VESTA vs. Control.

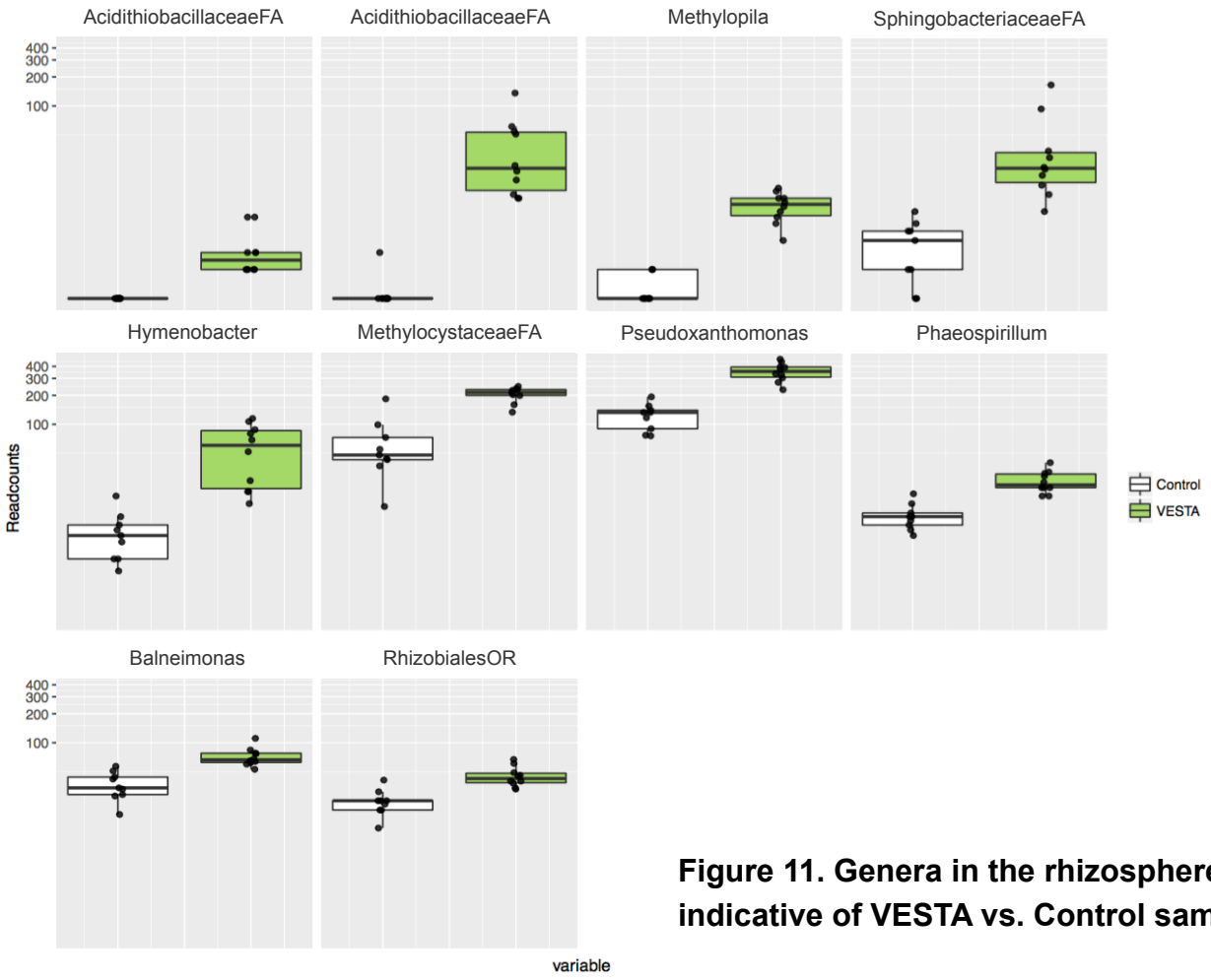
- VESTA treated rhizosphere contained several families that had higher read counts than Control samples.
- Two families with lower read counts in VESTA compared to Control are shown in the bottom panel.



Timepoint	Enriched in VESTA	Enriched in Control
2	Pseudoxanthomonas, Steroidobacter, NostocaceaeFA	Rhizobium, Pseudomonas, ActinomycetalesOR, Sphingomonas
3	Pseudoxanthomonas, Steroidobacter, NostocaceaeFA, Pseudomonas	Rhizobium, ActinomycetalesOR, Sphingomonas

Figure 10. Genera Enriched in the Rhizosphere of VESTA vs. Control Samples.

- **Pseudoxanthomonas** belongs to family Xanthomonadaceae from phylum Proteobacteria.
- **Steroidobacter** belongs to family Sinobacteraceae from phylum Proteobacteria. Steroidobacter are rod shaped bacteria that degrade steroids. Carrots are known to have phytosterols.
- **Nostocaceae** belongs to phylum Cyanobacteria. They are photosynthetic. Some of the species are known to have nitrogen fixing abilities, and may form symbiotic relationships with certain plants.
- **Pseudomonas** belongs to phylum Proteobacteria. The genus is well known for its metabolic diversity.



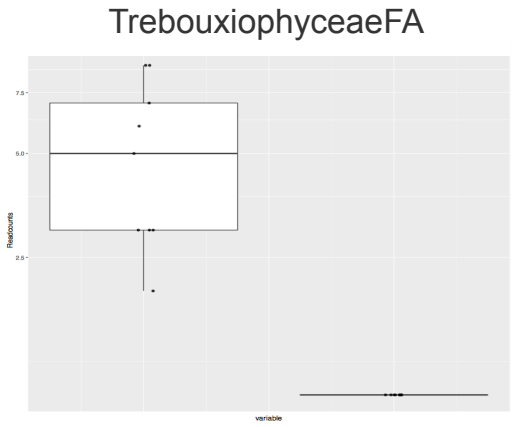


Figure 11. Genera in the rhizosphere that are indicative of VESTA vs. Control samples.

- VESTA treated rhizosphere contained several families that had higher read counts than Control samples.
- One genera with lower read counts in VESTA compared to Control are shown in the bottom panel.

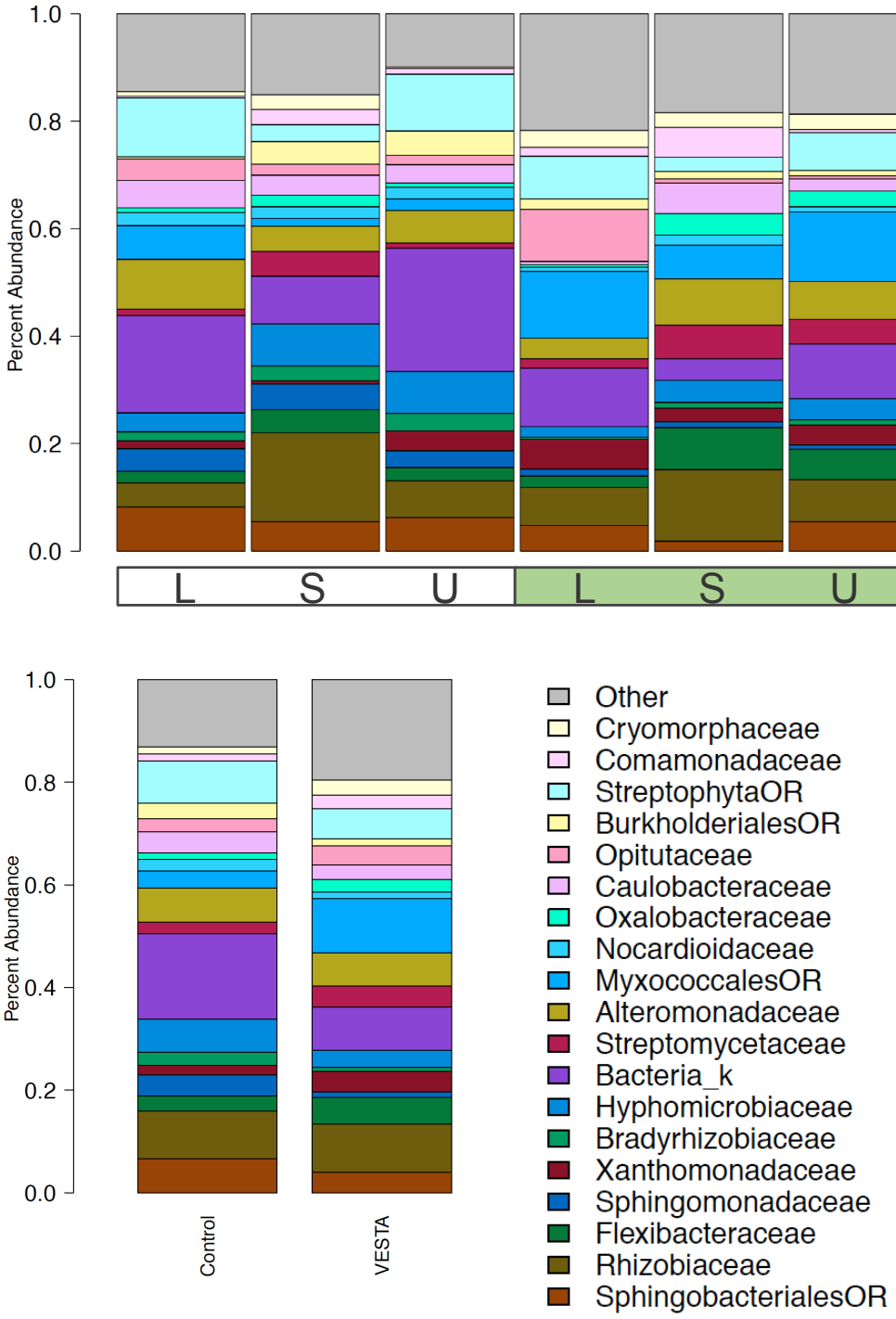


Figure 12. Family Enriched in the Root of VESTA vs. Control Samples.

- Each carrot root was divided into Shoulder (**S**), Upper (**U**), and Lower (**L**) sections (see Methods on page 4).
- Families enriched (Percent Abundance) in the three root sections are shown (top panel), and the overall VESTA vs. Control comparison is displayed (bottom panel).

Figure 12 Table: Family Enriched in the Root of VESTA vs. Control Samples.

Tissue	Enriched in VESTA	Enriched in Control
Shoulder	Flexibacteraceae, Xanthomonadaceae, MyxococcalesOR, Comamonadaceae	SphingobacterialesOR, Sphingomonadaceae, Hyphomicrobiaceae, Bacteria_k
Upper	Flexibacteraceae, Streptomycetaceae, MyxococcalesOR, Cryomorphaceae	Sphingomonadaceae, Bradyrhizobiaceae, Hyphomicrobiaceae, Bacteria_k
Lower	Flexibacteraceae, Xanthomonadaceae, MyxococcalesOR, Opitutaceae	Sphingomonadaceae, Bradyrhizobiaceae, Bacteria_k, Alteromonadaceae
Overall	Flexibacteraceae, Xanthomonadaceae, Streptomycetaceae, MyxococcalesOR, Cryomorphaceae	Sphingomonadaceae, Bradyrhizobiaceae, Hyphomicrobiaceae, Bacteria_k, BurkholderialesOR

- **Flexibacteraceae** was enriched in all three sections of the carrot root (see page 12 for more details about this family.
- **Sphingomonadaceae** was more abundant in Control samples, suggesting VESTA application reduced the abundance of this family in carrot roots.

★Members of Sphingomonadaceae are known to cause corky root disease of lettuce.
★In a previous study with lettuce, we found that Sphingomonadaceae family members were suppressed by VESTA application.
★These findings were correlated with significant reduction in lettuce disease in response to VESTA treatment.

These data suggest that suppression of **Sphingomonadaceae** family members is a common affect of VESTA treatment in lettuce and carrot fields.

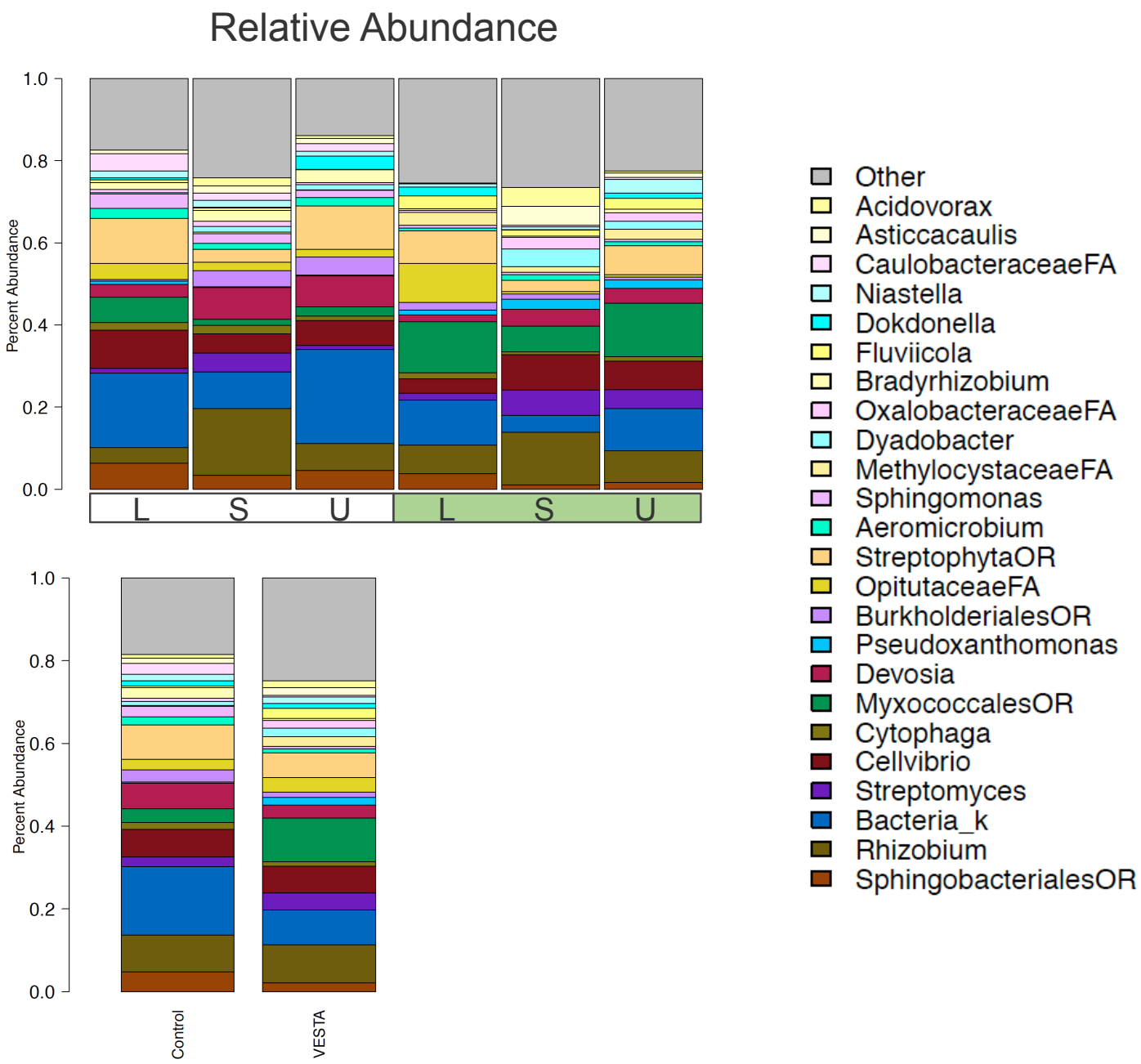


Figure 13. Genera Enriched in the Root of VESTA vs. Control Samples.

- Each carrot root was divided into Shoulder (**S**), Upper (**U**), and Lower (**L**) sections (see Methods on page 4).
- Genera enriched (Percent Abundance) in the three root sections are shown (top panel), and the overall VESTA vs. Control comparison is displayed (bottom panel).

Figure 13 Table. Genera Enriched in the Root of VESTA vs. Control Samples.

Tissue	Enriched in VESTA	Enriched in Control
Overall	MyxococcalesOR, Pseudoxanthomonas, MethylocystaceaeFA, Fluviicola	Bacteria_k, Devosia, BurkholderialesOR, Bradyrhizobium, CaulobacteraceaeFA

Genera enriched by VESTA in root endosphere	Genera suppressed by VESTA in root endosphere
Myxococcales belong to the phylum Proteobacteria. These bacteria move by gliding and typically act in swarms (*wolf packs*). Their cells are kept together by intercellular signals. They produce antibiotics and prey on other bacteria like *E. coli*. **YouTube Video: Myxococcus xanthus preying on an E. coli colony.** (https://www.youtube.com/watch?v=tstc6doiNCU)	**Bacteria_k** details TBD.
Pseudoxanthomonas belong to the phylum Proteobacteria.	**Devosia** belong to the phylum Proteobacteria.
Methylocystaceae belong to the phylum Proteobacteria. They are capable of using methane as the source of carbon and energy (methanotrophs).	**BurkholderialesOR** belong to the phylum Proteobacteria. Includes several pathogenic bacteria including species of **Burkholderia**, and **Bordetella**. (https://en.wikipedia.org/wiki/Burkholderia and https://en.wikipedia.org/wiki/Bordetella)
Fluviicola belong to the phylum Bacteroidetes.	**Bradyrhizobium** belong to the phylum Proteobacteria. Form symbiotic relationships with leguminous plant species to fix nitrogen in exchange for nutrients.
	CaulobacteraceaeFA belong to the phylum Proteobacteria. Species live in diverse habitats, mostly aquatic, also in soil, and at the surface of eukaryotes. They are chemo-organotrophic, some requiring peptone, B vitamins and amino acids (Abraham et al., 2014. In: The Prokaryotes: Alphaproteobacteria and Betaproteobacteria-- Rosenberg, et al. eds. Berlin, Heidelberg: Springer Berlin Heidelberg. 179-205).

Summary from online survey of genera enriched or suppressed by VESTA in the Root

- **Enriched**

1. **Myxococcales** with the ability to glide over and prey upon *E. coli* colonies in swarms.

- **Suppressed**

1. **BurkholderialesOR** that includes known pathogenic species.
2. **Bradyrhizobium** are nitrogen fixers for leguminous plant species. Carrots are not leguminous and may prefer other nitrogen fixers (see **Nostocaceae**, page 14; **Rhodospirillaceae**, page 12).
3. **CaulobacteraceaeFA** possible dwellers consuming nutrients (vitamins and amino acids) without benefit to carrots.

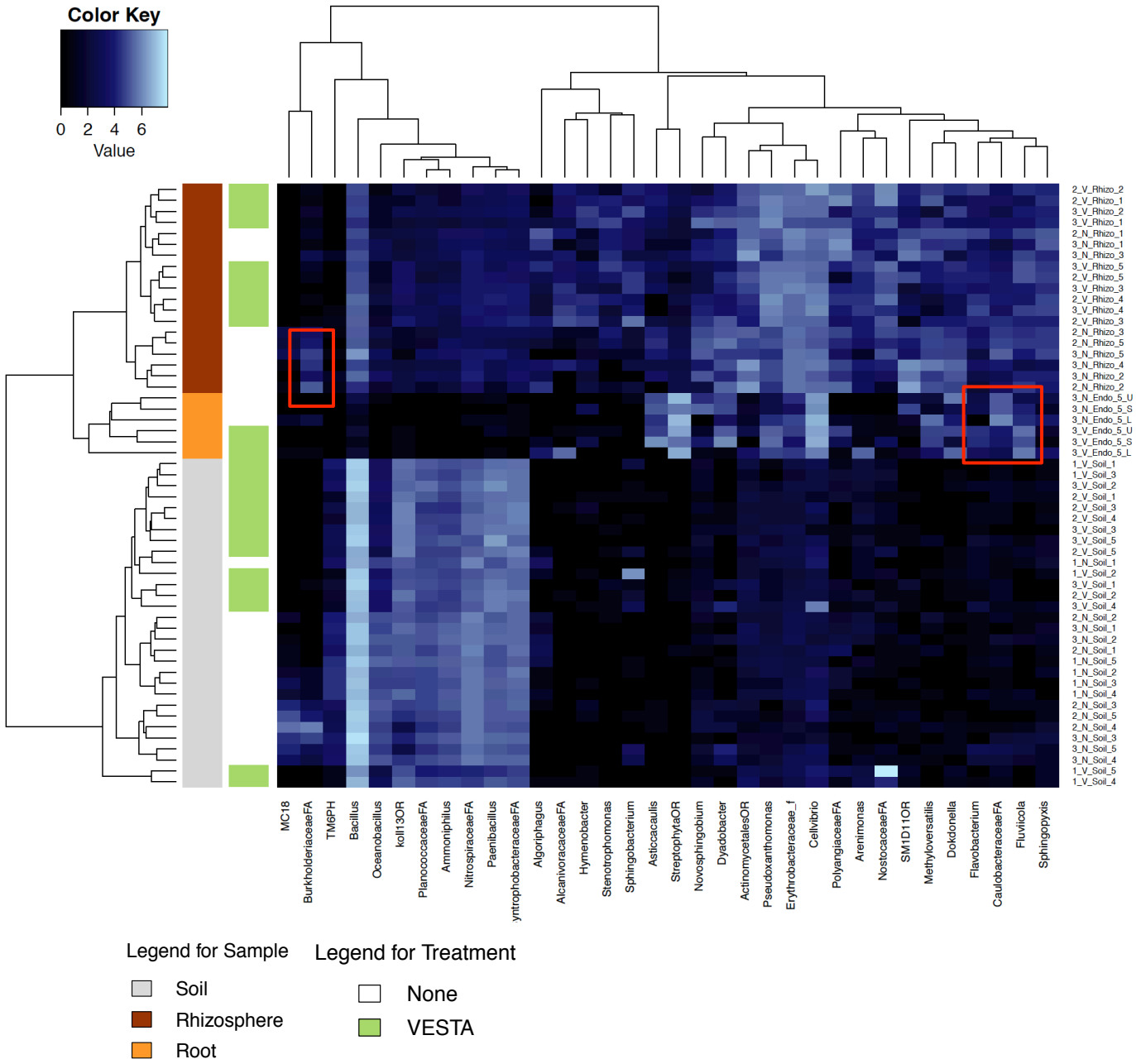


Figure 14. Hierarchical clustering demonstrates specific genera that are over or underrepresented in different sample types.

CONCLUSIONS

- Variation in community composition determined first by sample type, second by VESTA treatment, the effect of time point is not significant.
- There is a significant effect of VESTA treatment on microbial community composition in carrot; communities shift in response to VESTA treatment most dramatically in the root endosphere.
- Unexpected result: Microbial diversity (Shannon's Entropy) within the plant root and rhizosphere increases in response to VESTA treatment, while there is no significant change in soil.
- Root communities are the most affected by VESTA treatment of the three sample types investigated.
- VESTA Treated samples cannot be differentiated from Control samples at the Phylum level, but can be differentiated at family and genus level, suggesting that microbial community variation is affected by simple strain or species substitution.
- There is no unique OTU in plant root.
- The percentage of differentially present OTUs between VESTA and Control increases in Roots.

Standard Workflow For Each Sample (Standard Protocols)

- Described methods are adapted from earthmicrobiome.org, mobio.com, and opsdiagnostics.com.
- Protocols with customized modifications were applied to this study

1. DNA extraction from soil samples (mobio.com)

EXPERIENCED USER PROTOCOL

PowerSoil® DNA Isolation Kit Catalog No. 12888-50 & 12888-100

1. To the PowerBead Tubes provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. Check Solution C1. If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of Solution C1 and invert several times or vortex brie y.
5. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a at-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

Note

6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. CAUTION: Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean 2 ml Collection Tube (provided). Note
8. Add 250 µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean 2 ml Collection Tube (provided).
11. Add 200 µl of Solution C3 and vortex brie y. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean 2 ml Collection Tube (provided).
14. Shake to mix Solution C4 before use. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds. If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes. Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

10

15. Load approximately 675 µl onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the ow through and add an additional 675 µl of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature.

Note

16. Add 500 µl of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g.
17. Discard the ow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x g.
19. Carefully place spin lter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.
20. Add 100 µl of Solution C6 to the center of the white lter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x g.
22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). Solution C6 contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

2. DNA extraction from plant tissue (opsdiagnostics.com)

EXPERIENCED USER PROTOCOL

Materials Needed

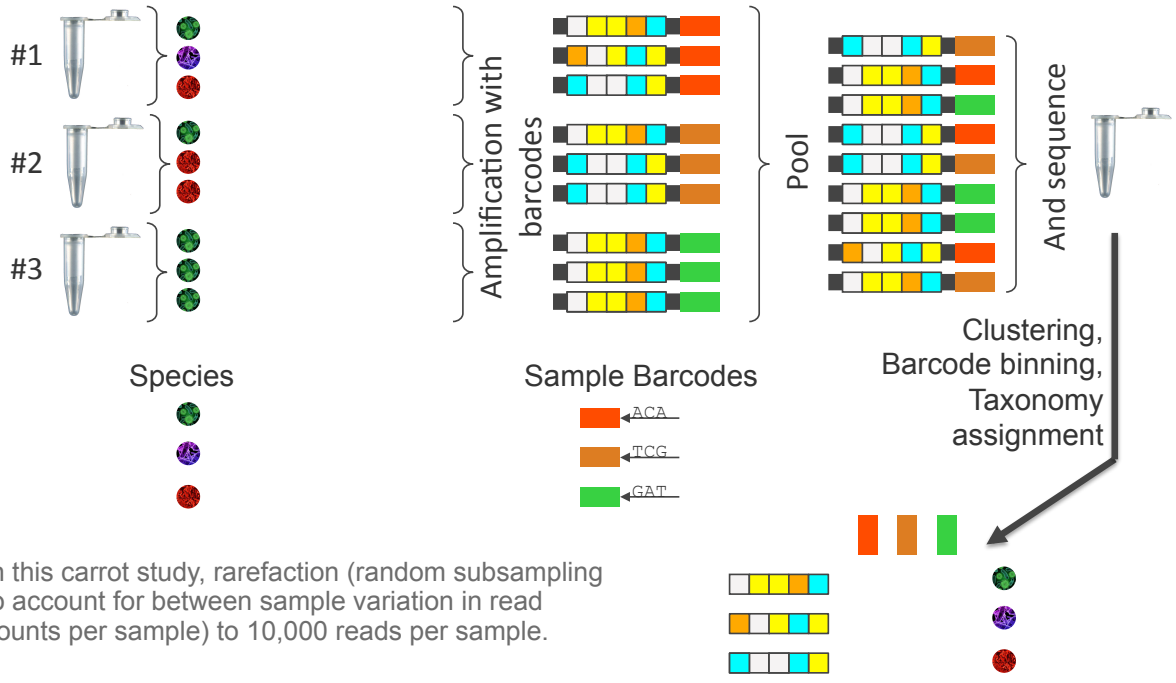
• CTAB buffer: 2% cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone,100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, or CTAB Extraction Buffer
• Centrifuge (up to 14,000 x g)
• Isopropanol
• 70% Ethanol
• 2ml centrifuge tubes
• SpeedVac
• TE Buffer (10 mM Tris, pH 8, 1 mM EDTA)

Method

Plant samples can be prepared by cryogenically grinding tissue in a mortar and pestle after chilling in liquid nitrogen. Freeze dried plants can be ground at room temperature. In either case, a fine powder is best for extracting DNA.

1. For each 100 mg homogenized tissue use 500 µl of CTAB Extraction Buffer. Mix and thoroughly vortex. Transfer the homogenate to a 60°C bath for 30 minutes.
2. Following the incubation period, centrifuge the homogenate for 5 minutes. at 14,000 x g.
3. Transfer supernatant to a new tube. Add 5 µl of RNase solution A and incubate at 32°C for 20 minutes
4. Add an equal volume of chloroform/isoamyl alcohol (24:1). Vortex for 5 seconds then centrifuge the sample for 1 min. at 14,000 x g to separate the phases. Transfer the aqueous upper phase to a new tube. Repeat this extraction until the upper phase is clear.
5. Transfer the upper aqueous phase to a new tube. Precipitate the DNA by adding 0.7 volume cold isopropanol and incubate at -20°C for 15 minutes.
6. Centrifuge the sample at 14,000 x g for 10 minutes. Decant the supernatant without disturbing the pellet and subsequently wash with 500 µl ice cold 70% ethanol. Decant the ethanol. Remove residual ethanol by drying in a SpeedVac.
7. Dry the pellet long enough to remove alcohol, but without completely drying the DNA. Dissolve DNA in 20 µl TE buffer (10 mM Tris, pH 8, 1 mM EDTA). The pellet may need warming in order to dissolve.

16S tag Library Amplification: Basic Concepts and Primer Design (Devin Coleman-Derr)



In this carrot study, rarefaction (random subsampling to account for between sample variation in read counts per sample) to 10,000 reads per sample.

3. 16S rRNA amplification with custom designed primers (earthmicrobiome.org)

EXPERIENCED USER PROTOCOL

Illumina PCR Conditions: 515f-806rB region of the 16S rRNA gene (Caporaso et al PNAS 2010):

Materials Needed

- PCR Grade H2O (note 1, below) 13.0 µL
- 5 Primer Hot MM (note 2, below) 10.0 µL
- Forward primer (10µM) 0.5 µL
- Reverse primer (10µM) 0.5 µL
- Template DNA 1.0 µL

Total reaction volume 25.0 µL

Method

Complete reagent recipe (master mix) for 1X PCR reaction

Notes:

1. PCR grade water was purchased from MoBio Laboratories (MoBio Labs: Item# 17000-11)
2. 5 PRIME HotMasterMix (5 PRIME: Item# 2200410) — NOTE: THIS PRODUCT HAS BEEN DISCONTINUED. WE ARE WORKING ON A REPLACEMENT.
3. Final primer concentration of master mix: 0.2 µM

Thermocycler Conditions for 96-well thermocyclers:

1. 94°C 3 minutes
2. 94°C 45 seconds
3. 50°C 60 seconds
4. 72°C 90 seconds
5. Repeat steps 2-4 35 times
6. 72°C 10 minutes
7. 4°C HOLD

Thermocycler Conditions for 384-well thermocyclers:

1. 94°C 3 minutes
2. 94°C 60 seconds
3. 50°C 60 seconds
4. 72°C 105 seconds
5. Repeat steps 2-4 35 times
6. 72°C 10 minutes
7. 4°C HOLD

Protocol:

1. Amplify samples in triplicate, meaning each sample will be amplified in 3 replicate 25 µL PCR reactions.
2. Combine the triplicate PCR reactions for each sample into a single volume. Combination will result in a total of 75 µL of amplicon for each sample. Do NOT combine amplicons from different samples at this point.
3. Run amplicons for each sample on an agarose gel. Expected band size for 515f/806r is roughly 300 – 350 bp.
4. Quantify amplicons with Picogreen (see manufacturers protocol; Invitrogen Item #P11496).
5. Combine an equal amount of amplicon from each sample into a single, sterile tube. Generally 240 ng of DNA per sample are pooled. However, higher amounts can be used if the final pool will be gel isolated or when working with low biomass samples. (Note: When working with multiple plates of samples, it is typical to produce a single tube of amplicons for each plate of samples.)
6. Clean Amplicon pool using MoBio UltraClean PCR Clean-Up Kit #12500 according to the manufacturer's instructions. If working with more than 96 samples, the pool may need to be split evenly for cleaning and then recombined. (Optional: if spurious bands were present on gel (in step 3), ½ of the final pool can be run on a gel and then gel extracted to select only the target bands.)
7. Measure concentration and 260/280 of final pool that has been cleaned. For best results the 260/280 should be between 1.8-2.0.
8. Send an aliquot for sequencing along with sequencing primers listed below.

IMPORTANT: Sequencing requires use of 16S and index sequencing primers and constructs.

4. Mapping 16S rRNA V4 Data to Reference Sequence Databases (earthmicrobiome.org)

- Select / develop a standard methods to map sequence database considering pros and cons.

Quality filtering and demultiplexing

1. Identify the first quality score below Q3 and truncate the read just prior to that position.
2. Determine if the truncated sequence is at least 75% of the length of the input sequence: if yes, retain the truncated read; if no, discard the read.
3. Determine if the truncated sequence has any N (i.e., ambiguous base call) characters: if yes, discard the read; if no, retain the read.

Closed-reference OTU picking

97% OTUs are picked in the initial EMP analyses using a closed-reference OTU picking protocol against the Greengenes database pre-clustered at 97% identify (this Greengenes reference collection build is available here: http://greengenes.lbl.gov/Download/Sequence_Data/Fasta_data_files/Caporaso_Reference_OTUs/gg_otus_4feb2011.tgz. This is done using pick_closed_reference_otus.py.

This process works as follows. Reads are pre-sorted by abundance in QIIME so the most frequently occurring sequences will be chosen as OTU centroid sequences. Each read is then searched against the Greengenes reference sequences using reference-based uclust version 1.2.22. The call to uclust issued by QIIME looks like:

```
uclust --id 0.97 --w 12 --stepwords 20 --usersort --maxaccepts 20 --libonly --stable_sort --maxrejects 500
```

Reads which hit a sequence in the reference collection at greater than or equal to 97% identity are assigned to an OTU defined by the reference sequence they match. Reads which fail to hit a reference sequence at at least 97% identity are discarded. Taxonomy is assigned to each OTU based on the reference sequence defining that OTU, and the Greengenes reference tree (also provided in the reference collection build linked above) can then be used for pylogenetic diversity analyses.

Closed-reference OTU picking has pros and cons, and future EMP runs will apply the open-reference OTU picking process described here: http://qiime.org/tutorials/open_reference_illumina_processing.html#option-2-subsampled-open-reference-otu-picking

The pros of closed-reference OTU picking are that it is fully parallelizable, which is important for data sets of this scale, and that the OTUs are defined by trusted reference sequences. It additionally serves as a quality control filter: erroneous reads will likely be discarded as not hitting the reference data set. The primary con of closed-reference OTU picking is that sequences that are not already known (i.e., represented in the reference data set) will be excluded.

5. Formatting of Sample Metadata to Comply with GSC Recommendations (earthmicrobiome.org)

The Biological Observation Matrix (.biom) file format is used for representing arbitrary biological sample by observation data matrices, such as OTU tables or metagenome tables, along with associated metadata. The .biom format allows for interoperability of "comparative -omics" software tools by providing a standard format that can be used across data types, and provides a convenient means for storing data and metadata in a single file.

6. Biological Relevance of data, literature surveys and functional determination (I-Cultiver speciality)

Scientific report with biological and functional relevance for product research and development. Recommendations for proprietary and regulatory filings. Development of next generation product.

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Strategic partnerships / collaborations:

- Dr. Devin Coleman-Derr, PGEC / USDA / U C Berkeley
- Dr. Ted Raab, Carnegie Institution For Science, Stanford University
- Dr. José Dinneny, Carnegie Institution For Science, Stanford University
- Dr. Fred Hempel, Baia Nicchia Farm
- Dr. Pamela Ronald, U C Davis
- Dr. Mohan Behl, Biotech. Park, Lucknow, India
- Dr. Todd De-Santis, Second Genome, Inc.
- TerreLocal (coming soon), a new way to find locally grown.

Important Disclaimer

All data presented in this report were produced from primary research following guidelines on good research practice.

For more information on this study, contact:

Rajnish Khanna, Ph.D.
Founder, CEO
I-Cultiver, Inc.
(707) 205-7084
raj@i-cultiver.com

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