Using next generation sequencing to describe epiphytic microbiota associated with organic and conventionally managed apples

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Abstract— *Its seems likely that agricultural management as diverse as certified organic and conventional IPM practices would impact the microbiota associated with crop surfaces differently. We sampled organic and conventionally managed apples at multiple time-points in a growing season and characterized the bacterial taxa associated with replicates of each treatment type. Surprisingly, no evidence of significant differences persisting across multiple time-points was observed. Significant differential abundance of certain taxa was documented but when it was, it was primarily associated with a single time-point making it difficult to understand if these observations resulted from an environmental or a treatment effect. Principal component analyses demonstrated that sampling time-point explained more of the differences between bacterial communities than treatment. Description of dominant shared bacterial families for both organic and conventional samples included Oxalobacteraceae, Pseudomonadaceae, Sphingomonadaceae and Enterobacteriaceae.*

Keywords— Organic, conventional, 16S, ITS, phyllosphere, bacteria, fungi.

I. INTRODUCTION

Organic agriculture is part of efforts to streamline agricultural practices to provide safer and more sustainably harvested foods. "Organic" became a certified practice with the passage of the Organic Food Production Act in the 1990 Farm Bill. For certified organic practices, the use of synthetic pesticides, genetically modified organisms, sewage sludges, irradiation, and other practices deemed to be detrimental to society and the environment are prohibited. While produce labeled ''organic'' means that a set of prescribed practices has been followed - these practices vary enormously by crop and geographic region. To understand what "organic" means for 5 different fruits and vegetables at any supermarket would involve extensive study of each commodity, where it was grown, weather pressures during the growing season, and what the regulations and approved materials may be for organic certification in each specific region.

Questions persist regarding how organic management may contrast with conventional management in terms of impact on harvest, plant pathology, and food safety. To better understand how organic and conventional management impacted an apple crop with regard to bacterial microbiota found on surfaces of fruits and leaves, we designed the experiment presented here. The microbial ecology associated with the surfaces of fruits and leaves is significant for all aforementioned considerations. For food safety, it is important to assess whether or not, a greater risk of exposure to human pathogens may correlate with organically or conventionally managed foods. Organic agriculture often relies on fertilizers or pesticides that are more natural materials - thus often supporting a more robust "native" microbial ecology that could be transferred to the crop. Fertilizers in particular host a wealth of bacterial and fungal species that may directly impact the safety of the crop especially if there is direct contact with edible surfaces of plants.

Here, we examine the impact of organic and conventional management on apples (cultivar Enterprise) grown in Queenstown, Maryland. Because so many different materials are used in the two management types (Table 1), we hypothesized that the organic and conventional fertilization and pesticide schedules would impact the epiphytic microbiota of the apple crop differently. Apple trees were grown in a randomized block design with five replicates of each apple tree under both certified organic and conventional IPM (integrated pest management) management. Trees were planted in 2003, with the first certified organic crop harvested in 2006. All samples for the work presented here were collected in 2008, after three consecutive years of certified organic and conventional management.

II. MATERIALS AND METHODS

2.1 Experimental Design of the Organic and Conventional Orchard

Randomized complete blocks of apple trees were planted (2003) in a one hectare (approx. 2.5 acres) plot at the Wye Research and Education Center in Queenstown, Maryland. Blocks were treated either with chemicals approved for certified organic management by the National Organic Program (NOP) of the USDA or with the most commonly applied chemicals in a standard commercial apple spray schedule for the Mid-Atlantic region (Table 1). Five replicates of each treatment were maintained for five years (2004-2008). Approximately 16 meters (50 feet) was maintained between plots to comply with Maryland Department of Agriculture regulation for proximity of conventional chemicals to certified organic lands (Figure 1). Certified organic materials from National Organic Program (NOP) lists were substituted at a one to one ratio with conventional materials used in a typical IPM management for commercial apples grown in the mid-Atlantic. The following organic and conventional materials were used to manage the orchard (Table 1).

TABLE 1
ORGANIC AND CONVENTIONAL MATERIALS

Treatment	Insecticides	Fungicides	Fertilizers	Bactericides	Herbicides
Organic	Kaolin, Pyrethrins, Spinosad, Azadirachtin	Copper, Sulfur	Kelp, fish emulsion, chicken manure, compost teas, 6-1-1 NPK 5-3-4 NPK	Streptomycin	Acetic acid, physical barriers (plastics)
Conventional	Pyrethroid, Carbamate, Organothio- phosphates	Carbamates	Calcium nitrate, 15-0-0 NPK	Streptomycin	glyphosate

Organic and conventional materials were substituted at a one to one ratio.

2.2 Enterprise Apples

The cultivar 'Enterprise' was used. Enterprise is a late ripening fruit that was bred in a cooperative breeding program of the Indiana, Illinois and New Jersey Agricultural Experiment Stations. Enterprise, like other disease-resistant cultivars has a field immunity to apple scab (*Venturia inaequalis)*, a high resistance to Fire Blight (*Erwinia amylovora)*, cedar-apple rust (*Gymnosporangium juniperi-virginianae)*, and a moderate resistance to powdery mildew *(Podosphaera leucotricha).* The letter "pri" in Enterprise commemorates the Purdue-Rutgers-Illinois cooperative breeding programs that contributed to the parental material for Enterprise and Goldrush cultivars.

2.3 Epiphytic Microbiota Sampling

At three time-points: (6/13 = 0) (6/18 = 5) (6/23 = 10) during the 2008 growing season, fruit and leaves of the cultivar 'Enterprise', were collected from 5 replicated blocks of organic and conventionally managed trees. Approximately 20 leaves plus two apples were placed in a sterile ziplock bag. Leaves were collected from around all sides of the tree and transported back to the lab in sealed bags in a cooler at 4° Celsius. Three hundred ml. of deionized water was added to the bags and samples were sonicated for five minutes to dislodge surface microbial species. The microfloral wash was transferred to centrifuge tubes and centrifuged at 30,000g for twelve hours at 4°C. Pellet was transferred to a small microcentrifuge tube and stored at –20°C until DNA extraction.

2.4 DNA extraction and 16S/ITS Amplicon Sequencing

The Promega Wizard DNA extraction Kit was used according to the manufacturer's specifications and 16S/ITS amplicon sequencing was performed according to Illumina's "*Overview of tailed amplicon sequencing approach with MiSeq*" protocol.

Forward primers were used with staggered nucleotides as described in Illumina's technical manual. This two-step PCR approach utilizes sequence specific primers and Nextera DNA Index Kit (Illumina, San Diego, CA). Sequence specific primers (IDT Inc., Coralville, Iowa) were designed according to low diversity amplicon specifications. Emerald Green GT PCR Master Mix (Takara Bio Inc. Otsu, Shiga, Japan) was used to generate amplicons. Clean PCR product was obtained using AMPure XT Beads to remove fragments smaller than 300 bases. Sample DNA concentration was determined using Qubit High-Sensitivity Assay (Life Technologies, Grand Island, NY). Samples were then diluted to 2 nM and pooled using 10ul of each sample. Ten microliters (10 ul) were taken from the amplicon multiplex sample and denatured with 10 ul .2 N

NaOH. This process was performed simultaneously for a 2 nM PhiX sample (Illumina, San Diego, CA) in a separate tube. Samples were incubated at room temperature for 5 minutes then 980 ul HT1 buffer (Illumina, San Diego, CA) was added to each sample to create a final concentration of 20pM. PhiX and amplicon multiplex samples were diluted to 5pM in 500 ul, and pooled together at a 1:1 ratio for a final volume of 1000 ul. Six hundred microliters (600 ul) of the combined sample was loaded on a MiSeq V2 cartridge (Illumina, San Diego, CA). Sample was sequenced on a MiSeq V2 platform.

2.5 Bioinformatic Methods

For 16S amplicon analysis, raw paired end fastq files output by the MiSeq platform were assembled using FLASH [1] with a minimum overlap criterion of 100 bp and maximum permitted mismatch density of 10%. The resulting merged sequences were filtered for quality and length (≥150bp) using QIIME [2,3], and spurious hits to the Phi X control genome were identified using BLASTN and removed. Passing sequences were trimmed for forward and reverse primers, evaluated for chimeras with UCHIME (*de novo* mode) [4], and subsequently filtered for host-related contaminant including chloroplast DNA and *Malus domestica* mitochondrial DNA using the RDP Bayesian classifier [5]. Finally a large-scale BLASTN search of the GreenGenes database (v13_05) was performed to identify unknown contaminant sequences. Identified contaminants included a substantial number of amplified mitochondrial DNA from fungal species largely reflecting the 12S rRNA gene region. The final set of cleaned 16S sequences was characterized for diversity and taxonomic composition using QIIME. Sequences were clustered into operational taxonomic units (OTUs) using UCLUST (*de novo*) [6] with a 95% identity threshold. Representative sequences of each cluster were assigned to a taxonomic lineage by the RDP classifier (trained on the GreenGenes 16S database, release v13_05) using a minimum confidence threshold of 0.80. Representatives were input to PYNAST [7] to generate a multiple sequence alignment, which was subsequently used to construct a neighbor-joining phylogenetic tree with FastTree [8]. After full characterization of the clean sequence dataset, sampling depth was normalized by randomly sampling 2,161 sequences from each sample. ITS paired end reads could not be assembled into consensus fragments due to insufficient length. Therefore we utilized the *R1* read set for our analysis, which consistently resulted in higher mean quality scores than the corresponding *R2* read sets. Reads were processed for length (≥150bp) and quality using QIIME, as well as spurious hits to the Phi X control genome. Passing sequences were trimmed for the *R1* associated primer and screened for chimeras with UCHIME (*de novo* mode). Passing sequences were searched against the UNITE ITS database (v12_11) to identify unknown contaminant DNA.

The final set of clean ITS sequences were characterized for diversity and taxonomic composition using QIIME. To provide even sampling depth, we first performed random sampling to 12,400 sequences per sample. Similarly to the 16S analysis, sequences were clustered into OTUs using UCLUST (*de novo*) with a 95% identity threshold, with OTU representatives assigned to a taxonomic lineage using the RDP Bayesian classifier trained on the UNITE db (v12_11) requiring a minimum confidence threshold 0.5. For both amplicon sets, alpha and beta-diversity metrics (e.g. Bray-curtis, unweighted UniFrac distance [9]) were computed in QIIME. Additional statistical analyses (e.g. hierarchical clustering and visualization) were performed in R (v.2.12.0) The false discovery rate (FDR) [10] was employed to control for false positives in comparative statistical testing.

III. RESULTS AND DISCUSSION

Dominant bacterial families observed in both organic and conventional samples included Oxalobacteraceae, Pseudomonadaceae, Sphingomonadaceae and Enterobacteriaceae (Figure 1). Interestingly, the family Lachnospiraceae was only observed in Conventional samples (Figure 1). Lachnospiraceae is a family in the order Clostridiales comprised of anaerobic species. Many genera in this family are commonly associated with mammalian intestinal microbiota. The use of this family has been proposed to serve as a fecal indicator [11]. The incidence of Lachnospiraceae in Conventional samples was restricted to only one time point (6/23 /10) so it may be difficult to establish if the occurrence of Lachnospiraceae was due to an environmental event, a contamination event or was indeed a result of some component of the conventional management.

Statistically significant differential abundance of certain bacterial taxa in organic compared to conventional treatments was observed at several different time-points, but not consistently across all the time-points. In the third time-point: (6/23 /10), a statistically significant ($P < 0.0098$) differential abundance of Cystobacterineae was observed in organic samples compared to conventional samples (Figure 2). Cystobacterineae are within the order Myxococcales. Taxa in this order have been described as widespread in terrestrial, ocean and soil environments[12]. Many have large genomes and are known for the formation of fruiting bodies and the ability to synthesize secondary metabolites [12,13]. Some taxa have been known to excrete hydrolytic enzymes that decompose complex biopolymers[12]. Another family that was significantly differentially

enriched (P< 0.011) in organic samples was Oxalobacteraceae. Oxalobacteraceae is a family of Betaproteobacteria in the order Burkholderiales [14] (Figure 2). Using principle component analyses to visualize differences in bacterial communities that may be correlated to treatment or sampling time-points revealed considerable overlap but also a small clustering effect of organic and conventional samples by treatment (Figure 3). A separation of early (red) and mid (green) sample time-points was quite pronounced but a later time-point (blue) was indistinguishable from the early time-point (Figure 3).

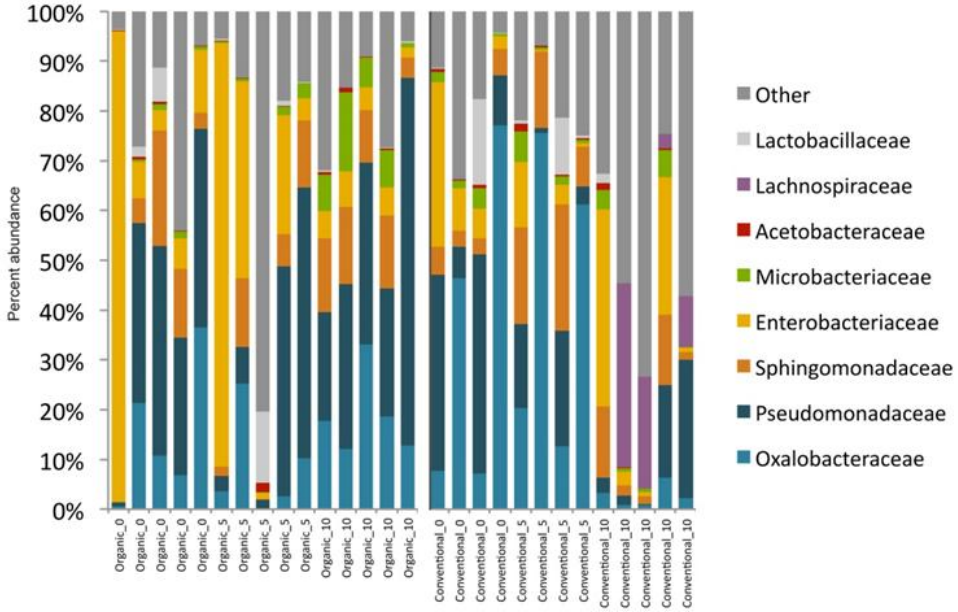

FIGURE 1 BACTERIAL FAMILIES IN ORGANIC AND CONVENTIONALLY MANAGED APPLES

Abundance of the most dominant bacterial families observed in both organic and conventionally managed apples across three time-points (0, 5, 10).

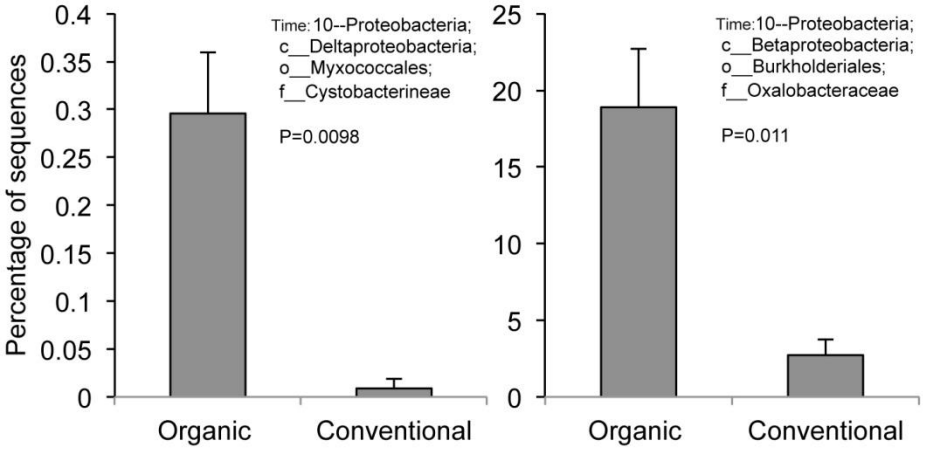

FIGURE 2 SIGNIFICANT DIFFERENTIAL ABUNDANCE OF BACTERIAL TAXA

Bacterial taxa that were found to be significantly differentially enriched in organic or conventional treatments are shown here. At time-point 10 Cystobacterineae (P< 0.0098) and Oxalobacteraceae (P< 0.011) were both significantly enriched in organic treatments.

Fungal taxa in organic and conventional samples over all sampling time-points were fairly consistent for both treatments. The most abundant fungal taxa in both treatments were Dothioraceae, Pleosporaceae, Hysteriaceae and Micosphaerellaceae (Figure 4). Several fungal taxa were significantly differentially enriched in both conventional (Capnodiales) (P< 0.00068) and organic (Tremellales) (P< 0.0037) samples (Figure 5).

It was interesting to observe that the majority of taxonomic representation in organic and conventional treatments were shared both for bacterial (Figure 1) and fungal (Figure 4) taxa. Previous work[15] documented more significant differences correlated to organic and conventional treatments, however conclusions were based on a very small number of sequences in comparison to the work presented here – which can exaggerate differences. Leff at al. documented differences between microbiota associated with organic and conventional spinach, lettuce, mushrooms, strawberries, tomatoes, peppers, peaches and grapes, however differences between organic and conventional apples was minimal[16]. Samples from the Leff et al. study were however exposed to many more variables than simply field management because samples were collected from point of sale.

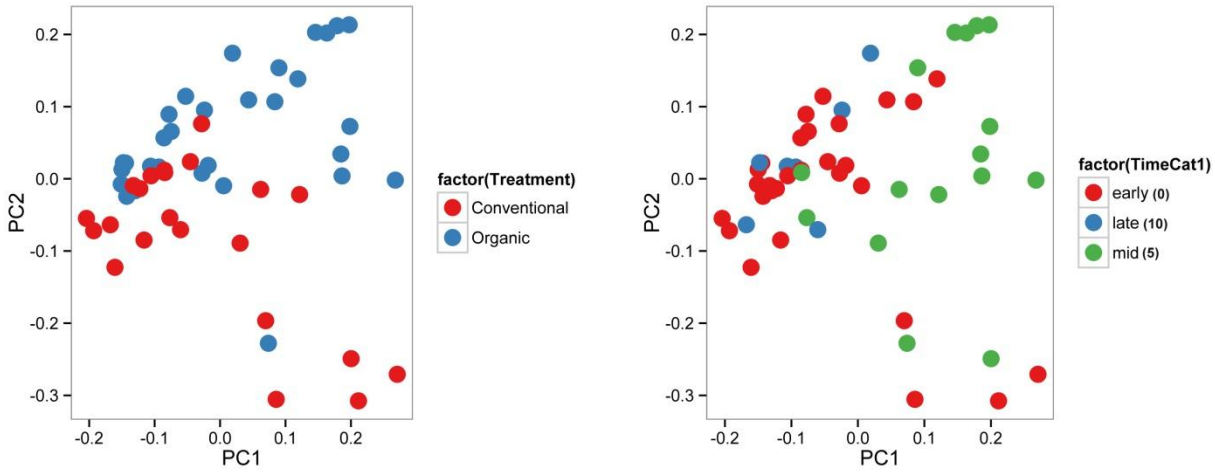

FIGURE 3 PRINCIPAL COMPONENT ANALYSES OF BACTERIAL COMMUNITIES IN ORGANIC AND CONVENTIONAL APPLES

A clustering by treatment is evident, potentially driven by differences associated with specific time-points. Early and mid time-points appear to cluster together while no separation is evident between early and late time-points.

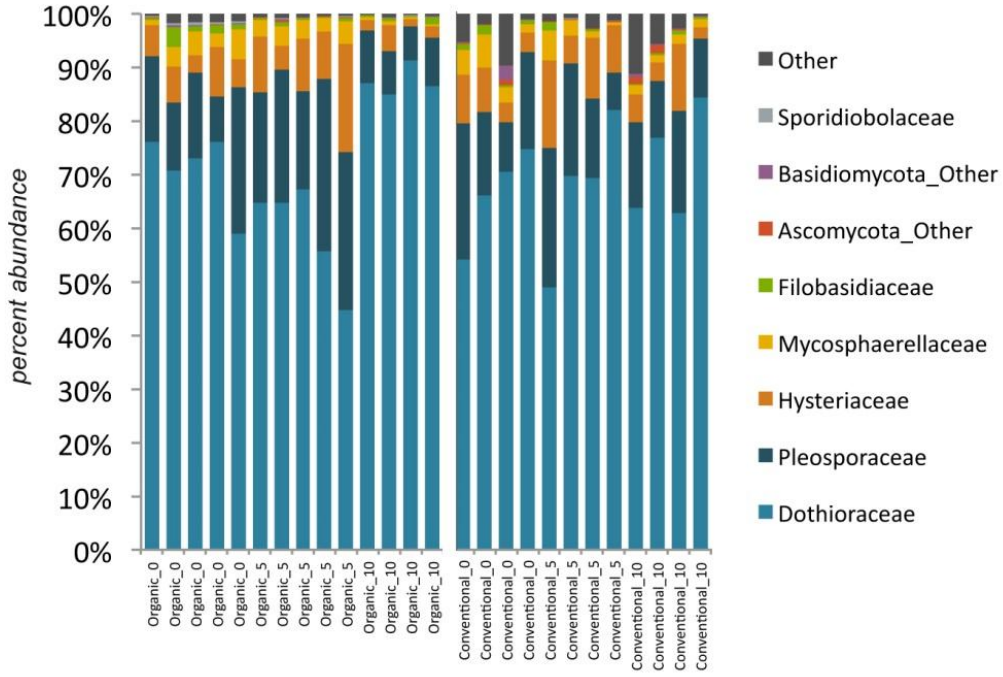

FIGURE 4 FUNGAL FAMILIES IN ORGANIC AND CONVENTIONALLY MANAGED APPLES

Abundance of the most dominant fungal families observed in both organic and conventionally managed apples across three time-points (0, 5, 10).

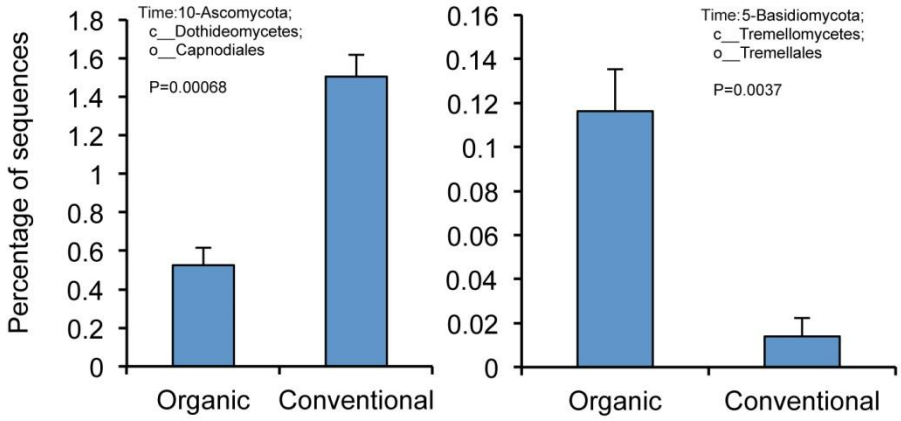

FIGURE 5: SIGNIFICANT DIFFERENTIAL ABUNDANCE OF FUNGAL TAXA

Fungal taxa that were found to be significantly differentially enriched in organic or conventional treatments are shown here. At time-point 10 Capnodiales is significantly enriched in conventional samples (P< 0.00068) and at time-point 5 Tremellales is significantly enriched in organic samples (P< 0.0037).

Whether or not actual field conditions and management practices play a significant role in the bacterial and fungal microbiota that are ultimately associated with fruits and vegetables when they arrive at the table is not fully understood. Although, much work has provided a growing assembly of "usual suspects" associated with microbiomes of agricultural commodities, the lack of the use of a control in most of these studies has limited our understanding of which microbiota are introduced by the environment, which microbiota may be host plant mediated and which microbiota may be influenced by agricultural practices such as organic and conventional management. A recent study [17] that employed inanimate surfaces as a control to study origins of phyllosphere microbiota found that controls and live plants shared almost the same taxonomic profiles – suggesting that environmental pressures may be strong enough to mask treatment effects introduced by practices such as organic and conventional management.

IV. CONCLUSION

Despite the drastically different agricultural inputs that were applied to the organically and conventionally managed trees, very few statistically significant differences were observed between samples from the two treatments and those that were, were associated with a single time-point, making it difficult to discern if it was a treatment effect, a temporal effect or an environmental effect. The high level of shared bacterial and fungal taxa suggests that environmental parameters (such as wind, dust, or air) may have had an equally strong influence on the epiphytic microbiota as the organic and conventional management did.

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REFERENCES

[1] Magoc T, Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27: 2957-2963.

[2] Kuczynski J, Stombaugh J, Walters WA, Gonzalez A, Caporaso JG, et al. (2011) Using QIIME to analyze 16S rRNA gene sequences from microbial communities. Current protocols in bioinformatics / editoral board, Andreas D Baxevanis [et al] Chapter 10: Unit 10 17.

[3] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7: 335-336.

[4] Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27: 2194-2200.

[5] Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73: 5261-5267.

[6] Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26: 2460-2461.

[7] Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, et al. (2010) PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26: 266-267.

[8] Price MN, Dehal PS, Arkin AP (2009) FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol 26: 1641-1650.

[9] Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R (2011) UniFrac: an effective distance metric for microbial community comparison. Isme J 5: 169-172.

[10] Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate - a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B-Methodological 57: 289-300.

[11] Newton RJ, VandeWalle JL, Borchardt MA, Gorelick MH, McLellan SL (2011) Lachnospiraceae and Bacteroidales alternative fecal indicators reveal chronic human sewage contamination in an urban harbor. Applied and environmental microbiology 77: 6972-6981.

[12] Brinkhoff T, Fischer D, Vollmers J, Voget S, Beardsley C, et al. (2012) Biogeography and phylogenetic diversity of a cluster of exclusively marine myxobacteria. ISME J 6: 1260-1272.

[13] Schneiker S, Perlova O, Kaiser O, Gerth K, Alici A, et al. (2007) Complete genome sequence of the myxobacterium Sorangium cellulosum. Nature biotechnology 25: 1281-1289.

[14] Garrity GM, Bell JA, Lilburn T (2015) Oxalobacteraceae fam. nov. Bergey's Manual of Systematics of Archaea and Bacteria: John Wiley & Sons, Ltd.

[15] Ottesen AR, White JR, Skaltsas DN, Newell MJ, Walsh CS (2009) Impact of organic and conventional management on the phyllosphere microbial ecology of an apple crop. Journal of Food Protection 72: 2321-2325.

[16] Leff JW, Fierer N (2013) Bacterial Communities Associated with the Surfaces of Fresh Fruits and Vegetables. PLoS ONE 8: e59310.

[17] Ottesen AR, Gorham S, Reed E, Newell MJ, Ramachandran P, et al. (2016) Using a Control to Better Understand Phyllosphere Microbiota. PLoS ONE 11: e0163482.