Community Consolidation Dictates Hydroponic Root Microbiome

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Abstract

Terrestrial plant roots mediate a complex network of prokaryotes and eukaryotes, collectively referred to as the rhizobiome. The ability to promote beneficial plant symbionts can contribute to increased crop productivity. Despite recognition of this microbial underpinning, plant cultivation strategies remain focused on plant physiology to determine crop health. The success of plant cultivation in hydroponics indicates that nutrient uptake, abiotic and biotic stress resistance is preserved in the rhizosphere regardless of the surrounding milieu. What has not been explored is how the microbial community adapts to soil-less hydroponic or aquaponic cultivation. Microbial dynamics in aquaponic systems are more complex than in hydroponics. As additional communities enter via the inflow water. This influx is shaped by microbial communities in the facility intake water, recirculating aquaculture system (RAS) environment including fish gastrointestinal tract, and the biofilter community of the soluble-waste treatment loop.

This study presents results from a series of experiments aimed at characterizing the development of the rhizobiome during the cultivation of Batavian lettuce (*Lactuca sativa*) in hydroponic or aquaponic systems coupled to freshwater RAS with common carp (*Cyprinus carpio*) or Nile tilapia (*Oreochromis niloticus*). In the first set of experiments, we employed standard water purification techniques (UV, ozone, H_2O_2) to impede microbial proliferation in hydroponic beds. This provided a macroscopic view of how sterilization processes impact rhizosphere and plant health.

In the second set of experiments we investigated the dynamics of rhizosphere colonization by foreign microbial communities such as the upstream RAS microbiome, a soil rhizosphere community, and probiotic supplementation. These were compared to treatments grown in sterilized nutrient solution. The similarity of taxonomic profiles across all treatments indicated that the plant roots strongly dictate the rhizobiome regardless of other environmental pressures or microbial influences. Upstream nitrifying microbial communities were not reflected in the rhizobiome – indicating that nitrifying organisms from the biofilter in the RAS do not carry over to the rhizosphere but rather are functionally replaced by rhizosphere-specific nitrifiers. Nonindigenous communities in proximity to plants are not capable of displacing the pre-existing rhizobiome in hydroponic nor aquaponic systems.

1 Introduction

The region in and around plant roots, commonly referred to as the rhizosphere, is a site of nutrient and electron exchange involving all kingdoms (1-6). The microbial component of the rhizosphere is referred to as the rhizobiome. It is composed of a core component fulfilling essential duties required by the plant at that instant, and a satellite component consisting of strains present at low abundances (7). The core community is stable across all plants and environments, consisting of taxa that thrive in the rhizosphere but not bulk soil (8). Recently, a consensus has been reached that plants exert a greater pressure in dictating their rhizobiome than do the microorganisms in choosing their hosts (7, 9, 10). The rhizosphere is an essential vector through which nutrient uptake needs (1, 11-13), abiotic stress resistance (10, 14, 15), and host defense are managed (16-18). Conceptually, the rhizobiome is a bidirectional exchange similar to the animal gut microbiota as an interface with the outside world. Not only do plants influence their rhizobiome, but the microbial community may directly influence biosynthesis processes in the plant (19, 20). As only 7% of bulk soil microorganisms are enriched in rhizosphere environments (21), the relatively carbon-rich environment of the rhizosphere appears to create a precursory selection pressure. Albeit less diverse, the relatively stable flow of 10-250 mg/g organic acids from the plant into the rhizosphere creates a microbial environment two orders of magnitude more abundant than surrounding soil (22). Apart from organic acids, these root exudates also include amino acids, organic anions and sugars (23-26). The complex dynamics surrounding the formation of these communities has given rise to a plethora of metagenomic studies on the rhizosphere, yet applying this knowledge to predict plant health has remained elusive (7, 10, 12, 27, 28).

[Figure 1 here]

Given the discrepancy in rhizosphere diversity across soil-less and soil-based cultivation strategies (figure 1), and given the growing amount of publicly available annotated genomic information (e.g. KEGG, JGI databases), a thorough characterization of the essential core rhizosphere functionality for plants of agricultural importance is an attainable target for this decade. Towards this goal, hydroponics and aquaponics present an attractive environment for this research. Soil-less cultivation shrinks the rhizosphere diversity without affecting plant-mediated nutrient uptake (28, 29). Physiochemical parameters may be more closely monitored as the lack of soil prevents competition from other plants, excludes nutrient transfer processes specific to the soil environment, and greatly reduces the risk of soil-borne disease (30, 31). Nonetheless, these systems are continuously exposed to environmentally persistent microorganisms (water- and airborne colonization). In comparison to axenic plant cultures, these ubiquitous organisms may reduce inoculation biases. Whilst evidence from several studies has indicated improved crop productivity in aquaponic systems over hydroponic counterparts (32-36), a consistent scientific explanation is still lacking. Recent studies focusing on the diversity of microorganisms in aquaponic systems have given rise to many hypotheses as to how the microbial community may lead to increased plant growth rates based on the increased abundance of chelating agents, cofactors, enzymes, or hormones facilitating nutrient bioavailability, either directly or indirectly (37-40). Causal factors leading to increased crop production in aquaponics remain speculative (27, 41), although involvement of the microbial community is implicit.

In order to investigate the capacity of plants to optimize their rhizobiome to suit specific growth demands, this study focuses first on determining whether the increased microbial load from RAS leads to better utilization of rhizosphere niches by measuring crop harvest parameters in hydroponic, aquaponics, and sterilized aquaponic treatments. We then investigate shifts in taxonomic diversity of the rhizosphere subjected to different microbial sources (hydroponics solution alone, coupling to RAS, probiotic addition, soil-based inoculation) to determine whether the rhizosphere is consolidating around a particular community, or diversifying as a function of different treatments.

This study presents rhizobiome consolidation dynamics from two perspectives critical to hydroponics/ aquaponics. Firstly, we investigate the relationship between microbial abundance and plant health (impact of sterilization on crop harvest). Secondly, we investigate the robustness of the rhizobiome in soil-less cultivation amidst pressures from foreign microbial communities.

2 Methods

Two experiments consisting of three deep water cultivation trials were carried out at the Wageningen UR Greenhouse Horticulture Unit (Bleiswijk, NL). The goals of experiment 1 were to a) compare crop harvest yields in an aquaponic system to standard hydroponic cultivation, and b) localize the microbial contribution to either the proliferation of microorganisms in the hydroponic unit (microbial load) or to abiotic sources (microbial exudates). Experiment 2 was designed to either discourage or diversify microbial communities in the rhizosphere. The community response was then measured through 16s rRNA sequencing and subsequent metagenomic analysis.

In both experiments, a recirculating aquaculture system (RAS) and a hydroponics (HP) unit were linked but decoupled (figure 2). Two fish species were used, common carp (*Cyprinus carpio*) (experiment 1) and Nile tilapia (*Oreochromis niloticus*) (experiment 2). Exaudio RZ 79-43 (Rijk Zwaan, NL) Batavian lettuce (*Lactuca sativa*) was grown hydroponically either in hydroponic basins (40 plants/ basin, 5 technical replicates) or boxes (3 plants/box, 3 technical replicates). Boxes were designed only for experiment 2 to create a controlled environment not otherwise possible with basin cultivation (figure 2). Boxes, insomuch as they were self-contained, provided better control over microbial communities available to the plants. They did not completely prevent bacterial transfer as growth conditions were not sterile, nor were seeds sterilized prior to planting. Each box contained a Styrofoam sheet floating on nutrient solution. Four microcentrifuge tubes filled with 2% w/v agar-agar (Sigma, NL) were inserted into the sheet. Seeds were placed directly into the agar. After two weeks, the least developed of the four plants was removed. Each treatment was conducted in triplicate. Aeration was supplied with a 2HP blower (Leister Technologies AG) for basins. Due to the few amount of plants per volume of nutrient solution, it was deemed unnecessary to aerate the boxes. This assumption was validated by dissolved oxygen (DO) measurements indicating similar oxygen levels compared to basins.

[Figure 2 here]

For all treatments, seeds were incubated in darkness overnight at 25°C (boxes fully wrapped in aluminum foil + plastic sheet for humidity and contamination control). Filter sterilized (0.22 µm) hydroponic nutrient solution (HNS) was added to each box at the beginning of cultivation and exchanged for the treatment-specific nutrient solution after two days. Nutrient solutions were replaced weekly. Rather than fully draining each treatment, half of the box volume was exchanged. This was done to better preserve the microbial community developing in each treatment. Basins were similarly subject to weekly nutrient exchange. Supplementation was done as necessary to maintain the following

approximate macronutrient composition (mmol/L): 15.0 NO_3, 1.5 NH_4, 5.0 K, 1.5 Na, 3.0 Ca, 1.5 Mg, 0.1 Si, 0.1 Cl, 1.5 SO_4, 0.5 HCO_3, 0.5-1.0 P. The following trace elements were also added (µmol/l): 20.0 Fe, 7.0 Mn, 5.0 Zn, 20.0 B, 0.5 Cu, 0.1 Mo. Parameters maintained most rigorously were pH (kept between 6-7) and electric conductivity (EC) (kept between 2-2.5). RAS water for crops was taken via the biofilter effluent. In a decanting tank identical to that used to make the HNS, nutrients were added to achieve similar concentrations as the HNS. Any sterilization occurred immediately prior to nutrient addition.

Nutrient solutions were added to the treatments weekly. In basins, plants were grown either in HNS or nutrient supplemented biofilter effluent (HNS-BF) as summarized by table 1. Here, we refer to HNS from two full crop cycle as mature HNS (HNS m), to emphasize the underlying assumption that it has formed a stable microbiome at that point. Three types of sterilization were chosen: UV exposure, H_2O_2 addition, and ozonation. For the UV treatment, a UV light (5W lamp, capacity: 2500 L) was applied for 48 h. Ozonation was done on site at the Aquatic Research Facility (Wageningen, NL). Hydrogen peroxide was applied at 150 ppm to the RAS effluent 5 days prior to nutrient supplementation. Finally, a *Bacillus subtilis* enrichment culture ($5x10^{11}$CFU/g stock, applied to achieve a final concentration of 2 mg/L) was added to the HNS once when the main root stem was ca. 2 cm long. *B. subtilis* is widely used in commercial aquaculture facilities for both fish and crustaceans (42, 43), as well as in hydroponics (44-46). A one-way ANOVA test was used to determine the differences in crop growth (crop fresh weight and root dry weight), with post-hoc Tukey test performed to assess significant differences between treatments.

In experiment 2, a first crop cycle was carried out exclusively in basins with half of the ten basins consisting of lettuce grown on HNS and the other half on nutrient supplemented biofilter effluent. After eight weeks, the crop was harvest and new seedlings were planted, keeping the original HNS/HNS-BF layout. Box trials were set up alongside the second basin crop cycle. The second crop cycle was terminated after six weeks. Treatments were done with three technical replicates and supplied with nutrient solutions as described in table 1. To make sterilized HNS or HNS-BF, freshly made nutrient stock solutions were filter sterilized (0.22 µm). The probiotic *B. amyloliquefaciens* ($5x10^{11}$CFU/g stock, applied to achieve a final concentration of 2 mg/L) was added to sterilized HNS and to unsterilized HNS-BF. Unlike the aforementioned *B. subtilis, B. amyloliquefaciens* has been developed as a probiotic in hydroponics but not RAS (47-49). As a plant growth promoting probiotic not present in RAS, there is minimal risk (in comparison to *B. subtilis*) that operational taxonomic units (OTUs)

identified from 16s rRNA sequencing corresponding to this bacterium could originate in the RAS rather than the applied formulation. Soil inoculum was sourced from a 2-month-old Batavian lettuce plant grown locally in potting soil. Care was taken to remove specifically soil with a high density of roots so that the rhizobiome would be transplanted into the hydroponics environment. Approximately 50 mg of soil was added directly to the boxes.

Table 1. Nutrient solutions used across treatments over all trials.

Experiment	Treatments	Description
1	HNS m	HNS recycled in basins
	HNS BF	Nutrient supplemented biofilter effluent
	HNS UV	UV sterilized, nutrient supplemented biofilter effluent
	HNS O_3	Ozone sterilized, nutrient supplemented biofilter effluent
	HNS H_2O_2	H_2O_2 sterilized, nutrient supplemented biofilter effluent
	HNS probiotic 1	*B. subtilis* inoculated HNS m
2	HNS m	HNS recycled in basins
	HNS BF	Nutrient supplemented biofilter effluent
	HNS s	Filter sterilized HNS
	HNS BF s	Filter sterilized nutrient supplemented biofilter effluent
	HNS probiotic 2	*B. amyloliquefaciens* inoculated HNS s
	HNS BF probiotic 2	*B. amyloliquefaciens* inoculated HNS BF
	HNS soil	HNS inoculation with soil + roots from Batavian lettuce grown in soil

Water samples during all three trials were analyzed weekly for nutrient concentrations, pH, and EC (Groen Agro Control, NL). Any nutrient deficiencies were corrected on a weekly basis. Mature HNS and HNS-BF nutrient solutions originating from the same stock solutions as the basins were used in the boxes.

During the first experiment, temperature (°C) in the hydroponics unit fluctuated between 14-24°C (trial 1, winter conditions) and 17-29°C (trial 2, summer conditions). During the second experiment the temperature of the hydroponic component was regulated to 16°C. As these experiments were conducted in greenhouses and not climate rooms, temperature regulation was controlled by an automated venting system and not through direct heating or cooling. Broad spectrum lighting was maintained at 200 µmol/s/m^2 for 16 h/day for all trials, although supplementation was not done for trial

2 (due to summer conditions providing adequate irradiation). Crops were harvested after 6 weeks in the basins. Measurements were taken for fresh and dry weight (oven-dried, 80°C for 48 h) of both the harvestable crop and the roots, respectively.

Community DNA was isolated from each treatment in experiment 2 for subsequent metagenomic analysis. Briefly, DNA was isolated from the roots of each technical replicate using the DNeasy PowerSoil Kit (Qiagen, Germany). For basins, root samples were taken randomly from three plants. For boxes, all plant roots in an individual technical replicate (entire box of three plants) were combined. Root samples were washed once with phosphate buffered saline (PBS) solution. DNA was subsequently isolated from the roots following kit instructions. DNA was also isolated from the fish tank water column, biofilter effluent, facility water source, and the rhizosphere of a terrestrial lettuce plant using the same kit.

Pikovskaya agar was used to screen for phosphate solubilizing bacteria (50). Siderophore activity was screened using Schwyn and Neilands universal siderophore detection medium (51). In short, root samples from HNS treatments (ca. 5 mg) were suspended in PBS solution and vigorously vortexed for 10 min. The solution was then plated across three plates. Strong phosphate-solubilizing or siderophore-producing colonies were detected by a color change in the medium resulting in a halo. Individual colonies were streaked onto new plates; individual colony forming units were then plated again to ensure purity. DNA from the resulting isolates was extracted, the identity of the isolates was confirmed by amplifying the V3-V4 region of the 16s rRNA gene and comparing the sequenced results to the NCBI database.

Purified DNA was sequenced using universal 16s rDNA bacterial primers (BaseClear B.V., NL). Sequenced OTUs were taxonomically identified by comparison with NCBI databases. Taxonomically identified OTUs were then screened against the KEGG reference database using a python script, wherein KEGG entries with annotated genomes were identified and key terms were pulled that were associated with specific genes of interest. The resulting three datasets (OTUs without KEGG annotated genomes, OTUs with KEGG annotated genomes and therein associated matrix of pulled genes of interest) were subsequently processed using an R script. Each dataset was subdivided into six data frames related to the taxonomic level. The first two datasets (OTUs alone and OTUs found in the KEGG reference database) were pre-processed to determine the statistical significance of the treatments. To visualize the dataset, species frequency, species occurrence, the relative frequency of occurrence, and the richness of species across treatments were plotted. The following diversity indices

were then calculated: species richness, Shannon entropy, diversity index and evenness, Simpson diversity index and evenness, and Pielou evenness. Different transformations were then applied to the data frames to determine which strategy best normalized the data to a linear distribution without warping. These standardization techniques included square root and logarithmic scaling, standardization by species maxima (Chord transformation), standardization by treatments (Hellinger transformation), and double standardization by species and treatments (Chi-squared, Wisconsin transformations).

Several OTUs were present above 1000 hits per treatment (visible as the total frequency of OTUs plotted across treatments, see supplementary materials figure 1). As the break from normal data to outliers was not clearly delimitated but rather tailed off, the data were run twice without modification and with a maximum threshold of 1000 hits for any individual OTU present, summed across all treatments. Once normalized, the data were sorted into dendrograms using two agglomerative clustering methods: nearest and furthest neighbor sorting. The nodal distance between objects in the dendrogram (cophenetic distance) was calculated for all object pairs (cophenetic correlation). The method giving the highest cophenetic score was determined to be the best clustering model for the calculated distance matrices. Next, the degree of membership of each object to its cluster (silhouette width) and the optimal number of clusters (silhouette optimal) were calculated for the original distance matrices. Binary matrices were computed from the dendrogram to determine the Mantel optimal number of clusters. These data were used to compute a reordered dendrogram considered to be the most robust clustering arrangement. This clustering arrangement was used as the backbone to create heatmaps of the species distribution across sites and as well the distribution of genes of interest across sites. Multiple factor analysis (MFA) was then used to generate heatmaps of the OTU distribution across treatments for each taxonomic rank.

Corresponding heatmaps were plotted, with the z-axis corresponding to intensities for the gene prevalence search results, OTU assignment on the y-axis, and treatments on the x-axis. As the gene prevalence is linked to the OTU assignment only, OTU/treatment combinations where the OTU was not present were first nullified. The gene prevalence was then plotted on the remaining OTU/treatment combinations. As 16s rRNA sequencing is relative in abundance, OTU abundance was considered in terms of presence/absence when making the plots.

3 Results

Results from experiment 1 indicated no significant difference in crop head weight between treatments. Nutrient supplemented biofilter effluent treatments or those inoculated with the *B. subtilis* enrichment culture had marginally greater root weights than the mature HNS treatment although this was not significant according to the ANOVA f-test at $p = 0.05$. H_2O_2 and ozone sterilization appear to have similarly marginally impacted root growth, whereas this trend was not observed with UV sterilization (figure 3). Nutrient supplemented biofilter effluent (HNS BF) treatments did not grow significantly better than other treatments. No significant difference was observed across dry root weights. Due to weekly supplementation, nutrients remained stable across both trials. Similarly, pH, DO, and EC were stable. Thus, sterilization of the RAS effluent does not seem to impact crop head weight.

[figure 3 here]

In experiment 2, we aimed to assess whether growth outcomes could be explained by a consolidation of the microbial community around a homogenous rhizosphere microbiome, nullifying treatment variations. Unlike previous trials in which plants were grown exclusively in basins, this trial employed the use of smaller boxes to allow a greater control over the microbial environment surrounding the plants. Sterilization treatments could thus be done with minimal cross-over between treatments by applying nutrient solutions separately for each box, likewise reducing contamination across technical replicates.

Extracted DNA passed all quality checks prior to sequencing. The Chao1 nonparametric richness estimator was chosen for this dataset due to the low abundance of most OTUs. Because 16s rRNA sequencing does not return true abundances, the number of OTUs detected in each sample (hits) is considered to be relative and thus valid only within this dataset. In total, 4232 individual OTUs were retrieved at the species rank, representing 896,948 hits across all treatments of which there were a total of 72,670 unclassifiable OTUs, meaning 8.1% of all hits were unassigned. The entire dataset was then screened against the KEGG database to identify annotated genomes. 1193 OTUs were matched against the KEGG database. 229 OTUs were matched but did not have annotated genomes and were rejected. To validate the representability of the 964 matched, annotated genomes, both KEGG-matched and unmatched OTU datasets were processed in R.

Species richness (Shannon diversity index) is higher in the outlier-removed, KEGG matching dataset compared to the unprocessed dataset (2.63 vs. 1.60). The Simpson diversity index increased along the same margin (2.27 vs. 1.57 for the outlier-removed and unprocessed dataset, respectively. As species

richness would only have decreased through data processing, this indicates a greater evenness across samples following data processing.

The cluster structure for both datasets was indistinguishable to five clusters corresponding to the following controls: soil, water source, and RAS or biofilter effluent, with all other treatments grouping in a fifth cluster. Interestingly, the hydroponic nutrient stock solution clustered with the hydroponic treatments. Clustering decisions were chosen based on a consensus between elbow, gap, Mantel, silhouette and NbClust (R package, bootstrapped 500 times) before being visually evaluated. Evaluating the NbClust algorithm with 50 bootstraps indicated convergence likely occurs much sooner, however this was not further investigated. Based on silhouette partitioning of the predicted clusters, phylogenetic diagrams were calculated based on Ward's method and plotted (52-54).

To visualize the distribution of OTUs across treatments, heat maps based on the above dendrograms were plotted. At higher taxonomic ranks, clustering becomes distorted due to the lack of input diversity. Conversely, at lower taxonomic ranks the data are unreadable due to the quantity of hits displayed. Due to the segregation used in generating the data (presence in KEGG database or not, removal of highly abundant (> 1000 hits) OTUs), the global clustering structure was assessed for each taxonomic rank and data subset. Importantly, global trends in the data were preserved regardless of taxonomic rank or subset. Figures 4-6 visually depict the clustering trends in the form of a cluster plot, dendrogram, and an unrooted phylogenetic tree at the genus rank. Supplementary materials figures 2a-c depict the same clustering at the phylum rank.

Figure 7 depicts the distribution of OTUs across treatments at the class rank. By viewing the same OTU distribution at several taxonomic ranks, the preservation of certain taxa becomes more apparent. As such, the reader is referred to supplementary materials figures 3a-d for heatmaps of the same distribution at the phylum, order, and family ranks. Due to issues in readability, lower ranks are not possible to visually interpret.

Ultimately, 22% of the uniquely identified OTUs had annotated genomes in the KEGG database. The distribution of gene and metabolic pathways amongst annotated genomes were plotted against OTUs and treatments allowing for a metric of total metabolic capacity. This was done for nitrogen, phosphorous, iron, potassium, and sulfur, as well as the abundance of specific genes encoding ammonia, nitrite, and nitrate. These were chosen as part of an exploratory project to evaluate the KEGG database as a tool for predicting gene expression capabilities based on 16s rRNA sequencing data. The

subset for nitrogen metabolism is depicted in figure 8; plots for other metabolic profiles of interest are provided in the GitHub repository provided by the link in the Data Availability section. The purpose of figure 8 is to visualize trends in the relative abundance of OTUs across treatments.

In addition to the described bioinformatics approach, we also plated water column and root samples on two selective agars to screen for strong phosphate-solubilizing or siderophore producing bacteria. Surprisingly, of the nine strains sequenced (table 2), all but two were of the genus *Erwinia*, the remaining two being unidentified *Enterobacter* species. Both genera fall into the same order of Enterobacterales.

Table 2. Bacterial isolates with the largest halo diameters when plated on Schwyn and Neiland medium (siderophore activity) or Pikovskaya medium (phosphate solubilization).

Selection medium for:	NCBI BLAST search results
Siderophore activity	*Erwinia persicina/ rhapontici*
	Enterobacteriaceae bacterium/ Erwinia sp.
	uncultured Erwinia sp./ Erwinia sp.
	uncultured Erwinia sp./ Erwinia sp.
	Erwinia rhapontici/sp.
	Erwinia rhapontici/sp.
Phosphate solubilization	*Erwinia endophytica/ sp.*
	uncultured Erwinia sp.
	uncultured Enterobacter sp.

[Figures 4-8 go here]

4 Discussion

Consolidation of the rhizobiome

This study presents the first concentrated metagenomic analysis of a microbial rhizosphere community in the hydroponic component of an aquaponic system. Whereas previous studies have employed sequencing techniques to characterize other compartments of aquaponic systems in detail (e.g. RAS tanks) or global microbial diversity across aquaponic systems (55-59), none have directly evaluated microbial dynamics in the hydroponic segment in response to upstream microbial pressures. Directly comparing yields between aquaponics and hydroponics has so far proven to be inconsistent and poorly reproducible (27, 41). As both aquaponic and hydroponic systems strive to maximize crop productivity through the same conventional means (greenhouse design, cultivar selection, etc.), they distinguish themselves primarily in their aqueous milieu, which although do not differ in availability of essential

nutrients (these are normally supplemented to a level where they are not limiting), may differ in terms of microbial abundance and diversity. Thus, our study focused on the community development at the main interface between the aqueous milieu and the plant – the rhizosphere. This research is furthermore relevant in relation to sterilization practices in aquaponics (e.g. with the goal of protecting crops from unwanted colonizers), an area that remains controversial. Some studies advocate for continuous cycling of water between RAS and hydroponic components (coupled aquaponics) (60, 61), while others have advocated for a discrete separation (decoupled aquaponics) with no return of microorganisms from the hydroponic component to the RAS (32, 62-66). We thus sought to determine whether sterilization (reducing the microbial influx into the hydroponics component) limits mainly adverse rhizosphere colonizers or rather all members of the community indiscriminately.

The rhizobiome is influenced by factors ranging from both the plant (genotype, life stage) and the environment (water source, nutrient profile) (7, 67), suggesting a highly dynamic community composition. Our results instead show a universal consolidation of the rhizobiome around a relatively consistent and narrow taxonomic profile (figures 4, 5, 6). This likely represents the combined core and satellite microbiome, as described previously by Yeoh et al. (2017) (8) and Compant et al. (2019) (7). As recently shown by Bartelme et al. (2019) (59), facility conditions strongly dictate the microbial populations present in RAS and aquaponic systems. Our results suggest that a similar facility-specific microbiome has formed in the hydroponic basins and boxes. Considering the RAS component as a pseudo-stable influence on the hydroponic microbial community, our results suggest that the unique microorganisms present due to the fish or biofilter do not directly shape the rhizobiome composition. Studies on the rhizobiome in other systems likewise indicate a similar pattern of consolidation. For instance, Schreiter et al. (2014) observed that the lettuce rhizobiome was consistent across varying soil types (68), while Edmonds et al. (2019) observed a rhizobiome unique from the circulating nutrient solution following 12 days of plant growth in aeroponic conditions (29). This trend appears to be a hallmark of all terrestrial plants (9, 10, 69, 70). Thus, it appears that selection pressures exerted by the plant to consolidate the rhizobiome around a particular profile are a fundamental aspect of plant physiology, preserved regardless the environment surrounding the rhizosphere. That profile, although observed as a collection of taxa, mirrors the functional needs required by the plant at a particular life stage and under particular environmental conditions.

Assessing rhizobiome resilience to foreign influences

The clustering models reveal several trends in microbial population dynamics. If the RAS were able to exert a significant pressure on the rhizobiome composition we would see hydroponic treatments supplied with nutrient-supplemented biofilter effluent clustering separately from those provided HNS alone - this was not observed. Pioneering bacteria originating from seeds are known to shape the next generation rhizobiome (16, 67, 71, 72). If the seed microbiome was strongly influencing rhizobiome development, we would expect the HNS stock solution to diverge from the treatments. Our data show that the HNS remains consistently clustered with the hydroponic treatments and always as a separate cluster from the other controls (water source, RAS, biofilter effluent). When clustering only hydroponic samples (excluding water source and RAS controls), we see some segregation by treatment (figure 4, supplementary materials). Probiotic samples cluster mostly as a single group (regardless of whether added to sterilized HNS or nutrient supplemented biofilter effluent treatments). The mature HNS community likewise groups together, with biofilter treatments (sterilized and non-sterilized) and sterilized HNS treatments grouping in another branch. The HNS stock, biofilter effluent, both the HNS and BF basins, and soil-inoculated treatments grouped along a single lineage, corresponding to the weakest grouping samples. Considering the HNS stock microbiome as a measure of the background microbial influence (environmentally persistent microorganisms), clustering was done with these OTUs removed (figure 5 a-c, supplementary materials). The same global clustering trend appeared, indicating that the HNS stock solution does not predetermine the rhizosphere community.

The clustering trends reveal that the nutrient profile of the hydroponics component is exerting a strong selection pressure on the pelagic hydroponic community, immediately reducing some of the upstream diversity. Additionally, however, clustering of sterilized treatments together with other treatments strongly suggest that the majority of root-colonizing bacteria are environmentally persistent bacteria already present at the facility and thus not defined by the specific water source. Further evidence for this hypothesis can be found when reviewing the soil-inoculated treatments. Community diversity was poorly retained when soil-based lettuce roots were used to inoculate sterile HNS. Unexpectedly, these treatments gravitated towards the same global consensus as the other hydroponic treatments - despite filter sterilization of the HNS and a lack of physical contact between treatments. This likewise supports the above claim that the HNS creates a selection pressure for environmentally-persistent, oligotrophic microorganisms able to thrive in relatively high nutrient environments (compared to the facility water source). Although the majority of the HNS stock microbiome is transferred to the hydroponics component, several taxa undergo major shifts in abundance during this transition. Figure 7 shows the shifts in taxa across treatments at the class rank, demonstrating a clear reduction in diversity between

controls (upstream communities, soil inoculum) and the hydroponic treatments. The hydroponic component microbiome is thus environmentally inoculated, water source independent, and capable of interacting with the rhizosphere to an extensive degree. It does not predict the rhizobiome, however, but more likely provides a pool of rhizobiome-candidates to colonize the rhizosphere when conditions favor their niche metabolisms.

Elucidating rhizobiome functionality

To elucidate the mechanisms by which coupling the hydroponics component to the RAS brings about a shift in the rhizobiome we first define the term "functionality" with respect to the role of microorganisms to plant health. It is tempting to interpret the aforementioned basal hydroponics microbiome as the core hydroponic-rhizobiome. This, however, leads to several issues. There is no consensus on which taxonomic rank is sufficient to declare an OTU assignment as definitively having a certain functional role in the rhizosphere. Framed otherwise, we ask whether is it sufficient to consider the entire genus of *Bacillus* as plant growth promoting, or all strains of the species *Bacillus subtilis*, or only certain strains? The region conserved among all known strains of a species, the pan-genome, can be quite low (40% for *Escherichia coli*) (73), creating diverse phenotypes at the subspecies level (74-76), in particular due to the frequency of homologous recombinant events (77). Thus, it is likely that the phylogenetic plasticity of microorganisms ultimately prevents any clear link between taxonomy and function. This is especially pertinent as the genomic resolution required to distinguish pathogens from plant growth promoters is impossible without whole genome phylogeny and likely as well *a priori* knowledge of potential pathogenicity (72, 75, 78, 79). Furthermore, some bacteria may be beneficial under certain conditions, yet can become pathogens when environmental conditions change (80). A detailed discussion on the phenotypic plasticity of symbionts has been covered by Pérez-Brocal et al. (2013) (79); case studies for certain common microorganisms can be found in Dobrindt et al. (2013) (75). In our study, plating organisms on selective media for phosphate-solubilization and siderophore activity allowed us to isolate several members of the *Erwinia* genus; a clade known to have versatile profiles ranging from plant growth promotion, rhizosphere opportunism, to obligate pathogenesis (81-85). That the strongest siderophore producing and phosphate solubilizing bacteria were all of the same order (Enterobacterales, table 2) is quite interesting, and is a topic that should be explored further. Evidently, functionality implies a state of transient symbiosis in which specific needs of the plant are accomplished by means of microbial interactions.

Other strategies are required to reveal specific functional contributions of organisms in the rhizosphere to plant health. Due to the combination of plant promiscuity in selecting its rhizobiome constituency and genotypic plasticity among microorganisms, we suggest the following two strategies as tools to assess rhizosphere dynamics: predicative functional assignment and transcriptomic validation.

We screened taxonomically assigned OTUs against the KEGG database. For each OTU, the sum of all genes surrounding a particular function of interest were calculated. The presence of OTUs across treatments was plotted, with the sum of genes incorporated as an intensity scale (figure 8). The goal of developing this tool was to more easily screen gathered taxonomic data for genes of interest that could be found in the KEGG database. This does not mean that the genes are being expressed, rather that they are likely present in the genome of the assigned OTU. Furthermore, this technique assumes the affinity of proteins transcribed from analogous genes assigned a particular function to be the same. This tool does not replace transcriptomic studies, but complements them (i.e. providing sequence information to aid in designing primers). Although likely best applied to identify the presence of specific genes of interest (e.g. enzymes, metabolites), we tested the method on our dataset for several generic metabolic pathways: nitrogen metabolism (subset into nitrite, nitrate, and ammonia related genes), phosphorous, iron, potassium, and sulfur metabolism.

There was no visible correlation between the presence of nitrogen metabolizing OTUs in the RAS or biofilter effluent and the hydroponic beds (figure 8). Thus, although nitrogen metabolizing OTUs dominate both the RAS and biofilter, those specific organisms do not appear to become integrated into the rhizobiome. While a subset of upstream OTUs do install themselves in the rhizosphere, the majority appear to originate elsewhere. As plants release considerable amounts of nitrogen (86) via exudates, these results may reflect changes in the concentrations of certain nitrogenous species proximal to the rhizosphere. It has been observed that bacterial communities in soil environments are sensitive to increased nitrogenous loads - reductions of up to 50% of total biomass and shifts in relative taxonomic abundances, with oligotrophic taxa particularly diminished (87-91). How these two selection pressures on the nitrogen metabolizing community of the rhizosphere counterbalance is unknown. Nonetheless, it appears that the oligotrophic RAS and biofilter nitrifying communities do not adapt well to the carbon rich rooting environment.

In an investigation into the effect of sterilization in the context of RAS coupling, Wielgosz et al. (2017) concluded that the beneficial effects on plant growth from RAS effluent were most likely conferred through microbial exudates, and thus unaffected by the sterilization process itself (36). While the

identity of those exudates remains unknown, our results further support their hypothesis by showing that the RAS microbiota does not play a significant role in colonizing hydroponic rhizobiomes.

Re-interpreting the effect of sterilization in hydroponic/aquaponic systems

We investigated if the method of sterilization impacts the integrity of exudates in the effluent. Hydroponic facilities recirculate the nutrient solution. Mainly done to improve water-use efficiency, retaining the microbial community is probably beneficial to plant health. Nonetheless, to discourage the proliferation of pathogens during nutrient recirculation, sterilization of the nutrient flow is often applied to varying degrees across facilities (92-99). For an overview of commonly employed sterilization techniques and their application in hydroponics, the reader is referred to the publication by E.A. van Os (2009) (97). In our study, ozone, UV-C irradiation, and H_2O_2 were tested as sterilization techniques. In terms of oxidation-reduction potential, ozone (2.07) is greater than H_2O_2 (1.78). UV-C radiation can interact directly with organic molecules, but may conversely precipitate trace metals (100). Conceptually, it was considered that from most to least destructive, UV would be followed by ozone and then H_2O_2 in terms of potentially detrimental effects on plant growth. Crop head and root weight measurements did not show consistent differences during the two harvests of experiment 1 (figure 3), nor did it appear that the method of sterilization significantly impacted the harvest outcome. The high variability among treatments suggested an increased precarity in the ability of the plant to resist stressors, which we explored further in experiment 2.

We then sought to investigate whether sterilization might serve as a preventative measure to curb latent opportunistic pathogens or heterotrophic bacteria competing for nutrients, and whether this would be reflected in differences in the rhizobiome composition. The singular role of sterilization is to add an additional diversity-reducing selection pressure alongside the hydroponic environment (pelagic, biofilm, and rhizosphere components). This selects for microorganisms that can thrive under the facility-specific conditions (temperature range, salinity, etc.). Of the hydroponic microenvironments, only the water-root interface is relevant to crop productivity. Specifically, taxa that may diminish crop production via proliferation in the rhizosphere are the main concern. One of the main mechanisms employed by plants in shaping their rhizobiome is commonly referred to as the ‘cry-for-help’ hypothesis (72), a phenomenon whereby the plant selects for microorganisms capable of alleviating specific stressors (nutrient deficiency, abiotic stress, etc.) (figure 9). The goal of sterilization must take this into account, and may thus be framed as “a mechanism by which the movement of latent,

466 waterborne pathogens are reduced to benign numbers". Unfortunately, this technique also results in a
467 reduction in the movement of beneficial bacteria. Given the slow growth requirements of k-strategists
468 (e.g. anammox (101-103) or archaea (56, 104)), system-wide population maturation likely is on the
469 order of months or longer (105). Maturation of the recirculating nutrient solution microbial community
470 is independent of individual crop cycles, as the same water is generally retained for subsequent cycles
471 (97). A mature hydroponics microbiome may improve plant stress response by increasing the amount
472 of candidate microbes for the rhizosphere as plant needs change. Outcompeting niches in the
473 rhizosphere likewise would limit the spread of r-strategists. If this hypothesis is proven, then circulation
474 in hydroponic systems may play a more centralized role than currently recognized in plant rhizobiome
475 resilience.

476 [figure 9 here]

477 In contrast to hydroponic systems, circulation in aquaponics does not typically rely on sterilization
478 between components; RAS water is not sterilized prior to entering the hydroponic unit. Thus, the
479 microbial community of the RAS always transfers to the hydroponic component. In coupled aquaponic
480 facilities, water is recirculated between the RAS and hydroponic components. Water travels
481 unidirectionally between the RAS and the hydroponic component in decoupled systems. Although
482 some forms of recirculation have been developed for decoupled facilities, they do not preserve the
483 microbial community present (for further literature on this topic the reader is referred to the book
484 *Aquaponics Food Production Systems* and related publications (63, 106, 107)). Regardless the
485 circulation system, a pseudo-steady microbial community will form in the hydroponic component
486 (referred to in this study as the mature microbiome). It is currently not possible to predict how long the
487 nutrient solution should recirculate within or between system components before a stable, resilient,
488 plant growth promoting community forms as discussed above. Furthermore, it would not be
489 unreasonable to expect successive waves of colonization to mark the maturation period, as is similarly
490 observed within the rhizobiome during root development (108-110). By comparing the maturation
491 process between aquaponic and hydroponic systems, future studies will be able to determine if the
492 increased diversity presented to the plants via their coupling to RAS allows for faster maturation of the
493 hydroponic component microbiome.

494 Finally, future rhizobiome maturation studies should prioritize research on active growth regions. Root
495 tips are the site of growth (similar to hyphal tips in filamentous fungi), and thus the main point of active
496 community interaction between plants and microorganisms (23). As such, they display a markedly

different community, becoming less diverse further away from the tip (10, 111). Root border cells – often considered to be the white blood cells analogue for plants in the rhizosphere – may persist outside of the roots for weeks or months and are known to interact with both pathogenic and nonpathogenic members of the rhizobiome (112-114). These unique cells, marked by highly dynamic transcriptomic profiles (114-116), can produce >90% of carbon-based root exudates (113, 115, 117). RNA-sequencing has been shown to be an effective tool in rhizosphere studies when assessing transcriptional dynamics (118-120). It is not known how root border cell expression or translocation differs between soil and soil-less culture. Nonetheless, given their important to terrestrial rhizobiome management, they likely play a non-negligible role in shaping analogous aquaponic and hydroponic microbial communities.

Microalgae – the rhizobiome storage chest?

A related, currently overlooked factor in these dynamics is the role of microalgae in relation to the rhizobiome. Microalgae are known to interact with heterotrophic bacteria through the excretion of organic carbon (121, 122). Furthermore, their presence is ubiquitous in water-based plant cultivation. Whether algal exudates can provide safe havens for rhizobacteria "on standby" remains to be determined. If the algal-bacteria relationship surrounding the transfer of carbon in exchange for nutrients or stress-alleviation indeed mirrors rhizosphere-bacteria relationships to a significant degree, it may explain the observed similarity between the HNS stock and hydroponics treatments.

5 Conclusion

Enhancing rhizobiome resilience and health has previously been described as a dual approach to increase crop yields while simultaneously improving plant survivability (72). The complexity of the rhizosphere environment as well the large datasets involved in metagenomic studies make discerning trends difficult. Our study confirms that rhizosphere consolidation around a relatively stable community composition occurs in both aquaponics and hydroponics. Given similar observations of rhizobiome consolidation in aeroponics and soil environments, this is likely a fundamental process for rhizosphere management by plants. Important for the field of aquaponics, this indicates that the bacterial community in roots is not directly modulated by the RAS or biofilter. The transfer of exudates and organic acids may play a role determining growth rates of plants in aquaponic systems, although no direct benefit appears to occur by means of changes to the microbiome. Our data suggest that the hydroponic environment does not hinder rhizosphere development, insomuch as the plant remains

dominant in shaping its rhizobiome. How exudate profiles and border cell adaptations to soil-less cultivation change in soil-less cultivation is still missing from this picture.

The fundamental trend that does appear to occur in hydroponic environments is a multi-step reduction in diversity by the operational parameters (e.g. facility conditions, use of sterilization techniques) and then by the plants themselves. Specifically, plants they exert a highly selective pressure on microorganisms entering the rhizosphere.

We re-iterate that taxonomic comparison is assumptive, as are conclusions about the interactions based on singular timepoints. The dynamic nature of microbial structure means that microbiome functionality assessments must be prioritized over cataloguing. As such, future studies would do well to focus on the functionality that certain taxa are providing in the system specifically in response to stressors in the rhizosphere (e.g. abiotic, pathogenic stresses). It is well established that this occurs at the root tip, and as our data demonstrate, it is a signal initiated by the plant. This is likely accomplished by changes in the quantity and diversity of root exudates in response to stressors on the plant. Genotype and life stage are likewise non-negligible influences on this dynamic.

Bacteria that thrive in the rhizosphere do not necessarily reflect the community of the RAS. This was demonstrated by mapping metabolic profiles (nitrogen, phosphorous, iron, potassium, sulfur) across treatments, indicating a divergence between the communities responsible for nutrient cycling in the fish tanks, biofilter, and hydroponic rhizobiomes. Future studies should couple predicative genotyping with transcriptomic analyses of the root tips. Linking the transcription of genes or pathways of known importance to shifts in the rhizobiome will inform crop management through a deeper understanding of how stresses impact plant-rhizobiome relationships.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

A.J. developed the theoretical formalism of the study. V.L. carried out the experiment with support from A.J., K.K., and J.B-M. V.L. performed the data analysis and wrote the manuscript with support from A.J. The final version of the manuscript received input from all authors. K.K. and A.J., supervised the project.

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10 Data Availability Statement

The datasets analyzed for this study can be found in the Git Hub repository: https://github.com/vonabol1/FiM2020Rhizobiome.git.

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