Spearmint enhances lettuce biomass and provides microbes to co-cultured lettuce in a decoupled aquaponic system

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Abstract

Aquaponics, where recirculating aquaculture systems (RAS) and hydroponics are combined to grow fish and plants, is a promising farming technique for sustainable food production. We investigated whether lettuce yield in aquaponics can be enhanced by companion plants and the role of companion plant-derived microbes. Lettuce and companion plants (spearmint, rucola, wormwood) were grown for 30 days in hydroponic units in RAS effluents in three time replicates. The growth of lettuce increased when grown with spearmint and rucola. Companion plants also influenced the bacterial community composition in lettuce. In particular, lettuce grown with spearmint or wormwood contained bacteria that potentially originated from the companion plant. A specific subset of bacterial taxa from spearmint were also associated with increased lettuce growth. On the other hand, the microbial community in mature lettuce did not develop markedly from the RAS effluents. However, the factor with the largest effect on plant growth and bacterial community composition was time replicate. In conclusion, the companion plants particularly spearmint enhanced the growth of lettuce through facilitation and potentially by providing lettuce with bacteria associated with increased growth. Our results emphasize the importance of the bacterial components and temporal fluctuations in optimizing aquaponics productivity.

Introduction

Aquaponics is a farming technique that combines farming of aquatic animals in recirculating aquaculture systems (RAS) and hydroponics (soilless plant production). In aquaponics, nutrients for plant growth are derived from the waste originating from feeding the aquatic organisms (Palm et al. 2018). Similar to other systems, the recycling of organically bound nutrients is governed by microbiological processes in aquaponics (Baganz et al. 2022). Compared to hydroponics, aquaponics may contain a more complex microbiome than hydroponics, because in aquaponics the plants use nutrient-rich effluents from recirculating aquaculture (Kasozi et al. 2021). The few studies of microbes in aquaponics have focused on the microbiomes in fish tanks, biofilters, biofilms (Eck et al. 2019; Kasozi et al. 2021; Schmautz et al. 2021), and fish faeces (Schmautz et al. 2017), and to a more limited extent in plant rhizosphere (Schmautz et al. 2017, Day et al. 2021). There is minimal information about how the nutrient-rich effluents from RAS and their microbes shape the bacterial communities in plants or how the variation of plant-associated bacteria impacts plant growth in aquaponics.

Microbial communities in aquaponics can influence plant production of the system positively or negatively (Eck et al. 2019; Joyce et al. 2019). Plant-associated bacteria may improve plant growth by decreasing abiotic and biotic stress and by affecting the absorption of nitrogen, phosphorus, iron, and other nutrients (Bacon and White 2016; Kasozi et al. 2021). On the other hand, proliferation of pathogens can harm plant growth (Stouvenakers et al. 2019). Further, pathogens from the RAS effluents could decrease the acceptability of the plants grown in aquaponic systems for human consumption. The microbiological safety of aquaponically produced food for human consumption is of interest both in terms of the fish (Joyce et al. 2019) and the plants (Elumalai et al. 2017).

A central aim in aquaponics is efficient nutrient removal from the RAS effluents by plants. Mixed plant species composition could improve the system efficiency (Van Ruijven and Berendse 2003) due to the complementarity of nutrient use by different plant species. Improved nutrient uptake in hydroponic systems with companion planting has been observed in practice (Geng et al. 2017). Efficient nutrient use is particularly relevant to aquaponic systems, because the removal of nutrients from the recirculating water is important for the fish wellbeing (Enduta et al. 2011; Yavuzcan Yildiz et al. 2017; Goddek et al. 2019). However, little attention has been paid to combining companion planting with aquaponics (Maucieri et al. 2017), while the microbes associated with the plants and from the environment are well recognized in soilless plant production (Senff et al. 2023). Companion plants may interact with each other by competition or facilitation (Brooker 2006), but also by suppressing harmful microbes and introduction of beneficial microbes (Zhou et al. 2023) or by increasing microbial diversity (Navrátilová et al. 2019; Liu et al. 2020).

Companion planting approach is traditionally used in terrestrial crop production systems and has gained increased interest recently (Tilman 2020). Companion planting has potential advantages such as higher productivity, improving defense mechanisms in plants, better disease control, enhanced ecological services, and economic profitability (Bomford 2009; Pickett et al. 2019). The combination of aquaponics with traditional plant co-culturing has potential to improve food production efficiency in an environmentally friendly way. Companion planting could improve plant productivity and quality via plant-plant and plant-microbial interactions (Li et al. 2020; Brooker et al. 2021; Lin et al. 2021). Soilless systems are excellent model systems to study such interactions as the systems are relatively simple and interaction pathways are immediate compared to systems with soil.

In this work, we used an experimental decoupled aquaponic system where lettuce and companion plants were grown together in nutrient-rich effluents from RAS. The study aimed to explore how companion plants from a range of taxonomic affiliations affect the growth and microbial community in lettuce (*Lactuca sativa*). We hypothesized that (i) lettuce growth is facilitated by companion planting due to higher bacterial diversity in lettuce when grown with companions and (ii) lettuce bacterial community composition is affected by companion planting. To resolve the relative importance of microbiome-mediated interactions, we tested (iii) to what extent lettuce yield is associated with lettuce bacterial community composition. Finally, we tracked down the origin of bacteria in lettuce and paid specific attention to the role of the RAS effluents and companion plants as potential sources.

Materials and methods

Experimental setup and sampling

The study was conducted in a decoupled aquaponic system where four hydroponic units with plants were combined with one RAS unit with rainbow trout (*Oncorhynchus mykiss*) and operated for 30 days. The whole experiment was repeated three times to serve as replicates in time. The hydroponic system consisted of four deep water culture (DWC) units (1 m × 1 m × 0.35 m) with a polystyrene floating cover with 30-mm holes for holding plant baskets. Before the aquaponics experiment, lettuce and companion plant seeds were germinated in a greenhouse (for details see Supplementary information). After the germination period, 15 seedlings of lettuce with 15 seedlings of companion plants were transplanted in each DWC. When lettuce was the companion plant as a control treatment, 15 + 15 lettuce seedlings were transplanted. Thus, in one DWC unit, we had one of the following treatments: (i) lettuce + companion plant spearmint (*Mentha spicata*) Lett-MINT, (ii) lettuce + companion plant wormwood (*Artemisia absinthium*) Lett-WORM, (iii) lettuce + companion plant rucola (*Diplotaxis tenuifolia*) Lett-RUCO, (iv) lettuce + companion plant lettuce (*Lactuca sativa*) Lett-LETT (control treatment).

Sixteen hours of light was provided to the plants during the whole experiment by using LED lights (King wua Bright®, China). Humidity varied according to the temperature (Fig. S1) but was over 50% during the whole experiment. The four DWC units were completely separate and did not interact with each other. At the start of the experiment, 135 L water from the RAS unit was given to each DWC and later 100 L water was given daily. Water was aerated in the fish tank, biofilter tank, sump tank, and each DWC unit. Each DWC was supplied with additional trace elements (1.25 ml micronutrient solution (Ingestad 2006) and macronutrients (2 g of K_2SO_4 and 1.6 g of $MgSO_4 \cdot 7\ H_2O$) daily.

The RAS unit consisted of a fish tank (1 m^3), swirl pool solid filter (500 L), fixed bed biofilter (500 L), and sump tank (500 L). The total volume of water in the RAS was approximately 2500 L. In the RAS unit, 30 rainbow trouts (*Oncorhynchus mykiss*) each of approximately 200–250 g in weight were stocked at the start of each time replicate. Fish were purchased from a commercial supplier in Finland. The fish were not used in any procedures and they only provided water to grow plants in the hydroponic systems. In the RAS water, the concentrations of total ammonia nitrogen (TAN), nitrite, nitrate, and pH were recorded twice a week while temperature was recorded daily (Fig. S2). Tap water was added to the RAS unit to replace the water given to the DWC units. The DWC and related tubing were disinfected with 90% ethanol at the start of each time replicate. The position of the treatments in the DWC units was randomized between the time replicates. The details of the operation of the aquaponic system are provided in Supplementary information.

Plants were harvested at the start and on the 30th day of each time replicate. Start point samples contained the whole seedling, i.e., root and shoot together due to the small size of the plants. For the endpoint samples, roots and shoots were sampled separately. Each plant sampling consisted of three replicates of five pooled plants.

Lettuce and companion plant dry weight (60 °C, 72 h) was recorded at the end of each time replicate. For bacterial community analysis, three samples of five pooled plants from each plant species were surface-washed with sterile water, and samples of approximately 100 mg of fresh weight were collected. RAS water was sampled by taking three 50-ml water samples on the day of the harvest from the sump tank of RAS and filtered through a membrane filter (Millipore, 0.22 µm pore size, Ø 47 mm). All samples were stored at – 80°C.

DNA extraction

DNA for bacterial community analysis was extracted from 100 mg of plant material with the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) by using PL1 lysis buffer. Samples were homogenized using two 3.2 mm chrome steel beads and approximately 0.6 g of 0.1-mm zirkonia-silica beads (Biospec) in an Omni Bead Ruptor Elite connected to an Omni BR-Cryo Cooling Unit with speed 4 m/s for 2 × 45 s under cooling. DNA from the water sample filters was extracted with the Nucleospin Soil kit (Macherey-Nagel, Düren, Germany). DNA was stored at – 80°C.

PCR amplification

Bacterial 16S ribosomal RNA (rRNA) gene PCR was carried out as a nested PCR to limit the co-amplification of plant chloroplasts and mitochondria. The primers in the first PCR step were 799F (5´-AAC MGG ATT AGA TAC CCK G-3´) and 1492R (5 ´-GGY TAC CTT GTT ACG ACT T-3´) (Chelius and Triplett 2001). The 1st PCR step was run with the reaction components 1 × GoTaq buffer, 0.2 mM dNTPs, 0.24 µM primers, 0.75 U GoTaq polymerase (Promega) and 2–10 ng DNA template with 25 µl of total volume per reaction. The cycling program was 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 53°C for 40 s, 72°C for 60 s and finally 72°C for 5 min.

The primers in the 2nd PCR step were M13-1062F (M13 linker for attaching barcodes and sequencing adapters 5’-TGT AAA ACG ACG GCC AGT-3’ (Mäki et al. 2016) followed by 1062F 5’-GTC AGC TCG TGY YGT GAG-3’) (Ghyselinck et al. 2013)) and 1390R (5´- ACG GGC GGT GTG TRC AA- 3´) (Zheng et al. 1996). In the 2nd PCR step, 2 µl of the 1st PCR product was used as a template with the same reaction components and cycling program as in the 1st PCR with 20 cycles.

Barcodes and sequencing adapters were added in a separate PCR step. The forward primer contained sequencing adapter IonA, barcode and M13 linker. The reverse primer was P1-1390R, where P1 is a sequencing adapter. The other reaction components and the cycling program were the same as in the 1st PCR but with 8 cycles and 2 µl of the 2nd PCR product as template. PCR products were purified with sparQ PureMag beads (Quantabio) and the DNA concentration of purified products was measured with Quant-iT PicoGreen dsDNA Assay (Invitrogen). The proportion of bacterial (ca. 350 bp) and plant mitochondrial (ca. 700 bp) amplicons in the purified PCR products were estimated based on image analysis of agarose gels with ImageJ software, and equimolar amounts of the products were pooled based on the bacterial product. The mitochondrial product was removed by gel extraction of the bacterial amplicon pool with Monarch DNA Gel Extraction Kit (New England BioLabs).

Sequencing and sequence processing

Pooled bacterial amplicons were sequenced using Ion Torrent PGM with Ion PGM Hi-Q View OT2 Kit, PGM Hi-Q View Sequencing Kit, and Ion 316 Chip v2. The sequencing data was analyzed with mothur software package v.1.43.0 (Schloss et al. 2009 following the relevant parts of MiSeq SOP protocol (https://mothur.org/wiki/MiSeq_SOP; accessed in April 2020; Kozich et al. 2013). Sequences were quality filtered using average quality of 25 and a window size of 50 bases, a minimum sequence length of 200 bp, a maximum length of 400 bp, maximum homopolymer length maxhomop = 8, maximum number of ambiguous bases maxambig = 0, maximum number of differences to primer sequence pdiffs = 1, and maximum number of differences to barcode sequence bdiffs = 0. The sequences were aligned against the Silva database v.1.38 (Quast et al. 2013). Chimeras were detected with UCHIME in de novo mode (Edgar et al. 2011) in mothur. After quality filtering, alignment, and removal of chimeras and nontarget sequences, 1485253 bacterial sequence reads were recorded. Pairwise distances of the sequence reads were calculated for the clustering step and operational taxonomic units (OTUs) were defined with a 97% similarity cutoff by the opticlust method. The OTUs were classified against Silva v. 1.38 to get their taxonomic affiliations. Bacterial richness (number of OTUs) and Shannon diversity were calculated in mothur with the command summary.single. One start sample, four companion plant samples, and 8 lettuce samples were removed due to few reads. The sequence data were submitted to NCBI under BioProject accession no. PRJNA814115.

Statistical analyses

Total plant production and lettuce biomass were calculated as the average weight of three composite samples consisting of 5 plants per species by subtracting the start weight from the end weight in each time replicate (n = 9). The total production of lettuce grown with lettuce was calculated the same way using three composite samples consisting of 5 plants per category (5 target lettuce and 5 companion lettuce) in each time replicate.

To assess the differences between treatments in total dry weight of lettuce (end lettuce weight – start lettuce weight) and total plant production in the systems, a two-factor analysis of variance (ANOVA) followed by Tukey's post hoc test was performed where time replicate was considered as a random factor and treatment (companion plant species) as a fixed factor. In the case the time replicate and the treatment effect interacted, a one-factor ANOVA followed by Tukey's post hoc test was performed, where data were split into time replicates and treatments were considered as a fixed factor. Root and shoot OTU numbers and Shannon diversity between treatments were compared with one-factor ANOVA followed by Tukey's post hoc test as above. The normality of variables was checked by analyzing Q-Q plots and histograms. The homogeneity of variance was tested using Levene's test. All statistical analyses related to biomass, OTU number, and Shannon diversity index were conducted using the software SPSS (version 26).

The community composition of bacteria was assessed at the OTU level. All the microbial community analyses were carried out in R (v. 3.6.1, R Core Team 2019) and RStudio. Plots were prepared with the package ggplot2 (Wickham 2016). The R package phyloseq (McMurdie and Holmes 2013) was used to prepare taxonomy plots. Bacterial OTU data were rarefied with the function rrarefy in the vegan package (v. 2.4.2, Oksanen et al. 2019) to the median of read counts (7366 reads per sample). For the samples with less than median reads, all the reads were included (minimum 559 reads). Only OTUs with more than 5 reads were included in the multivariate analyses. The differences in bacterial communities between treatments were tested with permutational analysis of variance (PERMANOVA, McArdle and Anderson 2001) with the function adonis2 in vegan with Bray-Curtis dissimilarity and 999 permutations for significance testing. Time replicate was used as a blocking factor in permutations. To check if the data meet the assumptions of PERMANOVA, multivariate dispersion was tested by the function betadisper. The microbial community differences between the treatments were visualized by non-metric multidimensional scaling (NMDS) plots with Bray-Curtis dissimilarities with the function metaMDS in vegan. To identify OTUs that were differentially abundant in lettuce grown with companion plants compared to lettuce grown alone, we used DESeq2 (v. 1.28.1, Love et al. 2014), ANCOM (v. 2.1, Mandal et al. 2015; Kaul et al. 2017) and ALDEx2 (v. 1.20.0, Fernandes et al. 2014). An unrarefied OTU table was used, and only OTUs that occurred in 25% of samples in each comparison (lettuce vs. lettuce with companion) were included. An OTU was considered differentially abundant when at least two of the three tools detected it. In DESeq2, the design was 'time replicate + treatment', and cutoffs of $p < 0.05$ and $\log_2$ fold change > 2 were used for differentially abundant OTUs. In ANCOM, the analysis included the time replicate as a covariate, and treatment was used as a group indicator for detecting structural zeros. In ALDEx2, we used 256 Monte Carlo instances and effect size > 1 to detect differentially abundant OTUs. Redundancy analysis (RDA) was performed on Hellinger transformed OTU data (rarefied, only OTUs with > 5 reads) in vegan to test for correlation between bacterial community composition and lettuce biomass with the time replicate as a conditional variable. The significance of the RDA model was tested as permutations with the function anova.cca. Biomass-affected OTUs were defined as OTUs with RDA axis 1 scores ± 2 standard deviations of the median score. To estimate the potential sources of microbes in lettuce, we used SourceTracker (Knights et al. 2011). Only OTUs with more than 15 reads in the rarefied OTU table were included. Companion plants, lettuce itself, companion plant and lettuce seedlings, RAS water, and DNA extraction and PCR controls were considered as potential sources of microbes, and lettuce grown together with companion plants as the sink.

Results

Lettuce growth and the effect of companion planting

In our experimental decoupled aquaponic system, lettuce biomass was significantly higher when grown with spearmint or rucola as compared to lettuce grown with wormwood or lettuce itself as the companion (Table 1). Plant species composition also affected total plant biomass production (combined biomass of roots and shoots of lettuce and companion plant). When spearmint was grown as the companion plant with lettuce, the average total production was higher compared to all other treatments (Table 1). However, the main effect of the companion plant treatment on lettuce biomass (Table 2) and total production (Table 3) depended on the time replicate. Because of this interaction, the data were split into time replicates to evaluate the effect of the treatments within each time replicate. In the first replicate, lettuce biomass was highest when grown with rucola ($F_{3,8} = 21.19$, $p < 0.001$) (Fig. 1A). In the second replicate, the lettuce biomass was highest when grown with spearmint, but the biomass did not differ significantly from the lettuce grown with rucola or wormwood. However, lettuce biomass when grown with spearmint was significantly higher than the lettuce grown with lettuce ($F_{3,8} = 16.63$, $p = 0.001$). In the third replicate, lettuce biomass was highest when grown with spearmint ($F_{3,8} = 28.59$, $p < 0.001$) when compared to all other treatments.

Table 1
Total production and dry weight (wt.) of lettuce (mean ± SD, *n* = 9) per deep water culture unit across time replicates.

Treatment[a]	Total production (g)[b]		Dry wt. lettuce (g)[b]	
Lett-MINT	9.05	± 3.56 b	5.56	± 2.44 b
Lett-WORM	4.55	± 2.15 a	3.42	± 0.92 a
Lett-RUCO	4.83	± 2.63 a	4.77	± 2.63 b
Lett-LETT	3.77	± 0.88 a	2.51	± 1.12 a

[a] Lettuce was grown with the companions spearmint (Lett-MINT), wormwood (Lett-WORM) rucola (Lett-RUCO), and lettuce (Lett-LETT). The plants were grown in a decoupled aquaponics system with rainbow trout for 30 days.

[b] Means with different letters differ significantly ($p < 0.05$).

Table 2
Two-factor ANOVA on total dry weight (wt.) of lettuce when grown with the companion species spearmint, wormwood, rucola, and lettuce in a decoupled aquaponic system with rainbow trout in three time replicates.

Dry wt. lettuce	df	Error df	F	p
Companion plant	3	6	1.74	0.25
Time replicate	2	6	2.62	0.15
Interaction	6	24	18.84	< 0.01

Table 3
Two-factor ANOVA on total production (lettuce + companion dry weight) when lettuce was grown with the companion species spearmint, wormwood, rucola, and lettuce in a decoupled aquaponic system with rainbow trout in three time replicates.

Total production	df	Error df	F	p
Companion plant	3	6	4.47	0.05
Time replicate	2	6	4.83	0.05
Interaction	6	24	12.13	< 0.01

The total system production was highest in all cases when lettuce was grown with spearmint (Fig. 1B). In the first replicate, however, the difference was not significant between lettuce grown with spearmint and rucola ($F_{3,8}$ = 11.69). In the second ($F_{3,8}$ =21.13, $p < 0.001$) and third time replicate ($F_{3,8}$ = 60.49, $p < 0.001$) total production with spearmint was significantly higher than in the other treatments (Fig. 1B).

Microbial community in lettuce and companion plants

We first compared bacterial community composition among all the plant species (lettuce, spearmint, rucola, wormwood) and found moderate differences among them (PERMANOVA R^2 = 0.13, p = 0.001) and between their roots and shoots (R^2 = 0.09, p = 0.001) (Fig. 2). We then compared the bacterial community of only lettuce among the treatments to determine the effect of companion plants. The lettuce bacterial community depended partly on the companion plant grown with lettuce both in lettuce shoots (R^2 = 0.13, p = 0.020) and roots (R^2 = 0.08, p = 0.001) (Fig. 2A). However, compared to the effects of companion plants and plant species, time replicate had the largest effect on the bacterial community both in lettuce (R^2 = 0.30, p = 0.001) and in the companion plants (R^2 = 0.18, p = 0.001) (Fig. 2B). The dominant bacterial orders in companion species and lettuce in all treatments were Burkholderiales and Rhodobacterales (Fig. S3). Minor groups in all plant species were the orders Sphingomonadales and Rhizobiales and the class Acidobacteriae (Fig. S3).

The companion plant did not affect bacterial richness or diversity in lettuce roots or shoots (Fig. S4). Bacterial richness and diversity were highest in the shoots of lettuce grown with spearmint, but these did not differ significantly from lettuce grown with other companions.

Using differential abundance analysis, we identified OTUs that were enriched in lettuce in the presence of a companion plant compared to lettuce grown alone. With spearmint as a companion, ten OTUs in lettuce roots increased in relative abundance, representing Bacteroidia, Alphaproteobacteria, and Gammaproteobacteria, and one OTU decreased (Fig. 3A). When the companion was wormwood, five OTUs were enriched in lettuce roots (Fig. 3B). No enriched OTUs were detected in the presence of rucola or in the lettuce shoots in any treatment.

Redundancy analysis was conducted to explore the connection between lettuce biomass and the bacterial community composition. A small but significant portion of bacterial community variation was linked with lettuce shoot biomass (4.8%, p = 0.013) and root biomass (5.1%, p = 0.012). In comparison, the time replicate accounted for 26.2% of the variation in bacterial communities in lettuce shoots and 50.7% in lettuce roots (Fig. 4A, B). We then investigated which subset of bacterial taxa was associated most strongly with lettuce biomass. Lettuce shoot biomass was positively associated with members of the family

Rhodobacteraceae, the genera *Rhodobacter* and *Hypomicrobium*, and OTUs representing unclassified bacteria, among others (Fig. 4C). Lettuce root biomass was positively associated with OTUs representing numerous taxa including the genera *Rhodobacter, Arcicella*, and *Pseudomonas*, the order Burkholderiales, the classes Rhodocyclaceae and Acidobacteriae and the family Comamonadaceae, and OTUs representing unclassified bacteria (Fig. 4D). Four of the OTUs associated with high lettuce biomass were also identified as those enriched by the presence of spearmint as the companion plant (Otu0017 / Rhodocyclaceae, Otu0018 / Burkholderiales, Otu0069 / Burkholderiales, Otu0073 / Hyphomonadaceae; Fig. 3).

Finally, we assessed whether the companion plants could be an important source of bacteria in the co-cultured lettuce. Although the majority of bacteria associated with lettuce when grown with companions were the same as when lettuce was grown with lettuce, a substantial share of the bacteria in lettuce could have originated from the companion plant species it was grown with (Fig. 5). In particular, lettuce grown with spearmint and wormwood had over 50% of bacteria similar to those in their companion plants. On the other hand, lettuce grown with rucola shared only a small portion of rucola-associated bacteria. Because a rather small portion of bacteria in lettuce could be associated with bacteria in RAS water, there appeared to be a negligible transfer of microbes from the RAS effluents to the mature lettuce (Fig. 5).

Discussion

Aquaponics is one of the most promising novel ways to sustainable food production (Timmons et al. 2018, Okomoda et al. 2023). Our study extends the understanding of this soilless plant production system functioning in two ways. Firstly, we characterized the plant-associated bacteria in a system where nutrients from the RAS effluents were utilized by hydroponically grown plants (decoupled aquaponic system) in multiple plant species. Secondly, we tested the efficiency of companion planting in aquaponic plant production. We show that the companion plants, especially the spearmint, contributed to specific microbial communities and increased the growth of the target plant lettuce. We tracked down a novel possible pathway of microbial interaction in aquaponics, i.e., microbial transfer between co-cultured plants.

Companion planting increased the growth of lettuce and total production of the system

Companion planting affected plant biomass production in a species-specific manner. Altogether, lettuce yield and total plant production were higher in most systems with companion plants in comparison to the systems with one species (lettuce) only. The strongest increase in biomass was observed when lettuce was grown with spearmint. It should be noticed that the spearmint in the spearmint-lettuce system was highly productive, but at the same time the spearmint increased lettuce biomass. This effect supports the idea that facilitative mechanisms overruled competition for nutrients as shown before in some companion plantings. For instance, menthol mint (*Mentha arvensis)* has been shown to increase the productivity of wheat crop (Kumar et al. 2002). Peppermint (*Mentha piperita*) co-cultured with tomato resulted in higher biomass yields than tomato monocultures (Kumar et al. 2002). Peppermint (*Mentha x piperita*) has been shown to boost the defensive mechanisms in members of the Brassicaceae family when grown as a companion (Sukegawa et al. 2018). In contrast, in the systems with rucola, lettuce growth was enhanced, but rucola was not productive, which could be explained by the low inherent growth rate and poor competitive ability of rucola. Therefore, companion planting showed good potential to increase lettuce yield in aquaponics, but selecting the right companion is important.

Previous studies in plant-soil systems systems have shown that companion planting may increase productivity and alter the composition of secondary metabolites in plants (Ahmad et al. 2020). Members of the genus *Mentha* (Mimica-Dukic and Bozin 2008) and rucola (Bennett et al. 2007) are promising sources of secondary metabolites, and it is possible that lettuce growth was affected by secondary metabolites exuded by spearmint and rucola. Furthermore, root exudates contain primary metabolites which can enhance biological activity in root-associated microbes (Kuzyakov et al. 2007). Therefore, companion plants may affect target plant microbiome indirectly via secondary metabolites exuded by plant roots (Jacoby et al. 2020). Furthermore, the companion plant may be the source of the microbes itself (see below). These results warrant further research into the potential pathways spearmint and rucola may boost companion productivity.

Lettuce bacterial community composition was affected by the companion plant

Bacterial community composition in lettuce depended partly on the companion species supporting our hypothesis that companion planting affects plant microbiome composition. It has been shown previously in soil-based systems that the root-associated bacterial community differed in two pea varietes when grown together vs when grown in monocrops (Horner et al. 2019). Here we show further that spearmint and wormwood both potentially transmitted specific bacteria to the co-cultured lettuce in aquaponics. To our knowledge, there is little previous evidence of plant-plant transfer of microbes, and such interactions could be particularly important via water in soilless systems. Of specific interest is that some of the bacterial OTUs associated with spearmint were also associated with increased growth in lettuce. The same was not observed in the wormwood-associated OTUs. This finding suggests that the potential of a companion plant to promote lettuce growth depended not only on the plant species, but also on the specific bacteria associated with the companion plant. To maximize plant productivity in aquaponics, attention should be paid to the plant-associated bacteria in the system or even manipulating these bacteria to increase productivity.

Microbial communities and sources in the target plant lettuce

In line with the other research showing the importance of compartments in plant microbiome (Navrátilová et al. 2019), the microbial communities in lettuce root and shoot were distinct. In all our study systems, bacterial communities in lettuce were dominated by Burkholderiales and Rhodobacterales. Burkholderiales have been detected previously in lettuce grown in aquaponics (Stouvenakers et al. 2020). Bacteria in hydroponically grown plants most likely originate from the seedlings or the growth environment. In aquaponics, using RAS effluents as a nutrient source adds a rich source of microbes and could potentially mediate the transfer of also harmful microbes to plants, making them unsuitable for human consumption. Therefore, from a practical point of view, it is important to investigate whether microbes are transferred from the RAS effluents to the plants grown in aquaponics. The present study revealed that the bacterial community in mature lettuce did not develop markedly from the RAS effluents. It has been shown in a soilless system that the plant played a more important role in determining the rhizosphere microbial community than the microbes in water (Lobanov et al. 2022). Our results support the findings of Lobanov et al. (2022) as in our system the microbial communities in the lettuce were affected by the co-cultured plant species, but showed negligible transfer of bacteria from the RAS effluents to the lettuce. However, further experimentation on the transfer of microbes from the other compartments of an aquaponic system to plants is needed to generalize these findings.

Bacterial community composition but not diversity drove plant productivity

We hypothesized that lettuce growth is facilitated when co-cultured with another species due to higher bacterial diversity in the co-culture. Diversity and richness of plant-associated microbial communities can depend on environmental abiotic and biotic factors (Afzal et al. 2019). In soil, plant diversity may affect soil bacterial community composition and diversity (Schmid et al. 2021). In addition to the general relationship between species diversity and ecosystem productivity (Van Ruijven and Berendse 2009), bacterial diversity in tree leaves has been associated with plant productivity at ecosystem level (Laforest-Lapointe et al. 2017). In our system, the presence of a companion plant did not increase bacterial richness and diversity in lettuce although lettuce biomass production increased. Instead, we could relate lettuce biomass to the variation of a relatively small group of bacterial OTUs. Therefore, it was not the richness or diversity of bacteria overall, but the presence and fluctuations of specific taxa that were important to plant performance. A similar pattern of specific microbial assemblages but not diversity being related to plant diversity and productivity has been observed in root-associated fungi (Maciá-Vicente et al. 2023). The specificity in microbial-plant relations is traditionally acknowledged in negative interactions where pathogens are often specialized (Barrett et al. 2009, Wang et al. 2019). Recently, also the positive interactions are known to be microbe-specific, and mycorrhizal fungi (Bever 2002), endophytic bacteria (Afzal et al.2019, and rhizosphere bacteria (Compant et al. 2010) may promote host performance in a species-specific manner. Working with hydroponically grown melons, Lin et al. (2021) showed that the relative abundance of bacteria in the genus *Chryseobacterium* had a strong correlation with fruit weight, supporting our results of specificity of plant-microbial interactions in soilless systems. The bacterial taxa that were positively associated with lettuce biomass in our study have been associated with the growth of plants previously. For example, *Pseudomonas* species have been shown to enhance growth in tomato (Gravel et al. 2007). Members of the genus *Hyphomicrobium* are known as plant-associated methylotrophs (Macey et al. 2020), but in contrast to this study, in a previous study their occurrence correlated negatively with plant growth (Franke-Whittle et al. 2015). However, several *Hyphomicrobium* species are denitrifiers (Martineau et al. 2015) and another presently plant production-associated genus *Arcicella* has been connected to nitrogen and phosophorus cycling (Chai et al. 2017). These bacteria are good candidates as indicators of efficient nutrient transfer from RAS effluents to plants.

System production and bacterial community composition varied in time

The pre-experimental growth period and the aquaponics experiment were performed in three time replicates. We made all attempts possible to keep the environmental conditions comparable and stable during the pre-experimental and experimental phases. Nevertheless, we recognize the effect of daily variation in weather, tapwater quality, and possibly random drift. The large fluctuation among the time replicates suggests that variations in environmental factors such as temperature and perhaps random effects had a fundamental effect on plant growth and microbial assembly and that deterministic biotic interactions were relatively minor. However, against the background of strong temporal variation of the bacterial community, specific bacteria were connected to plant productivity. This makes comparing results from different experiments challenging. Companion planting may improve productivity of aquaponics, but the high variation in time replicates should be taken into account in planning applications and commercial production.

Concluding remarks

We conclude that companion planting facilitated lettuce growth in a decoupled aquaponic system. The facilitative mechanisms include the potential transfer of specific bacterial taxa from a companion species to lettuce, whereas the diversity of the bacterial community associated with the plant was not a major factor. The strength of the effect on lettuce growth depended on the companion plant species, which highlights the importance of selecting a suitable companion. Our results additionally emphasize the importance of the bacterial components and temporal fluctuations in optimizing aquaponics productivity. Further studies are required to fully understand the complex ways co-culturing may interact with the functionality of bacteria-plant and plant-plant interactions in aquaponic systems. The overall facilitative effect can be a collective effect of complex interactions, and novel experimental approaches are needed to tease these apart.

Declarations

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Competing Interests

The authors declare no competing interests.

Author Contributions

All authors contributed to the to the research design, research implementation and data analysis. Experiment and acquisition of data were performed by Faiqa Atique. The 1st draft of the manuscript was written by Faiqa Atique. All authors contributed in the writing of the manuscript. All authors read and approved the manuscript.

Data availability statement

The sequence data were submitted to NCBI under BioProject accession no. PRJNA814115.

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Figures

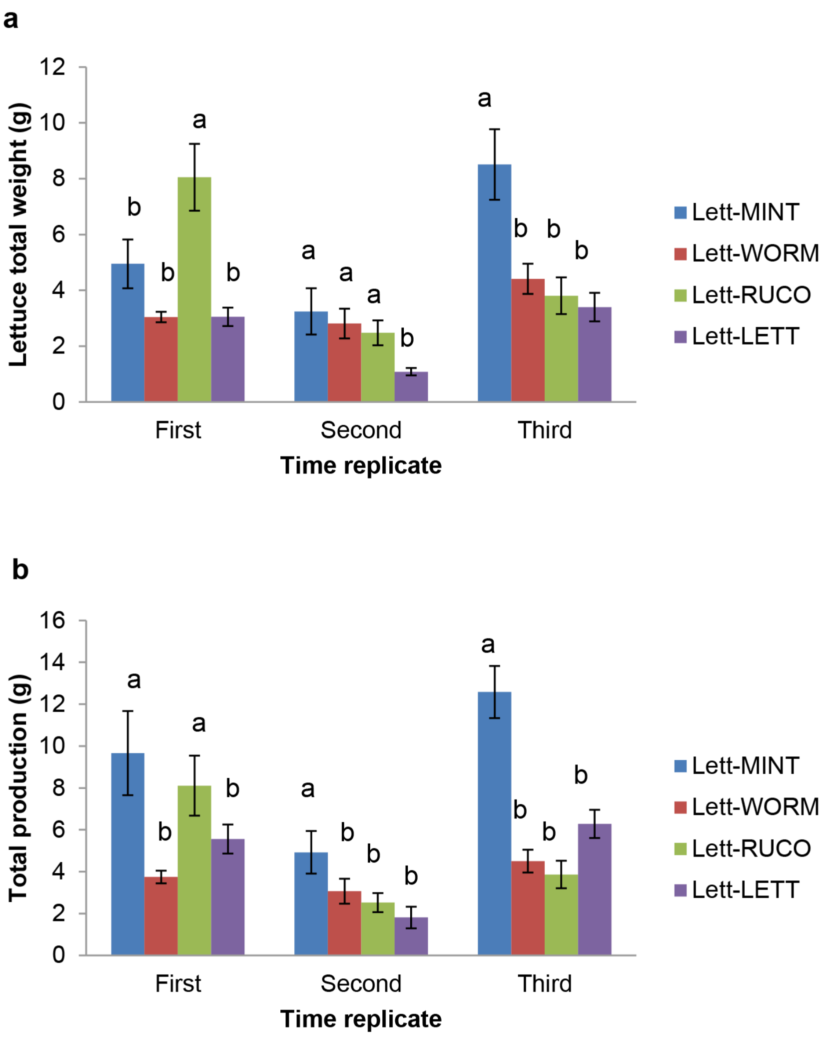

Fig. 1 Plant growth in a decoupled aquaponic system with companion plants. A) Total dry weight of lettuce grown with companion plant and B) total production (lettuce + companion) per deep water culture unit in the three time replicates (mean ± SD, n=3). Lettuce was grown with the companion species spearmint (Lett-MINT), wormwood (Lett-WORM) rucola (Lett-RUCO), or lettuce (Lett-LETT) for 30 days. Bars with different letters differ statistically significantly ($p < 0.05$) within a time replicate

Figure 1

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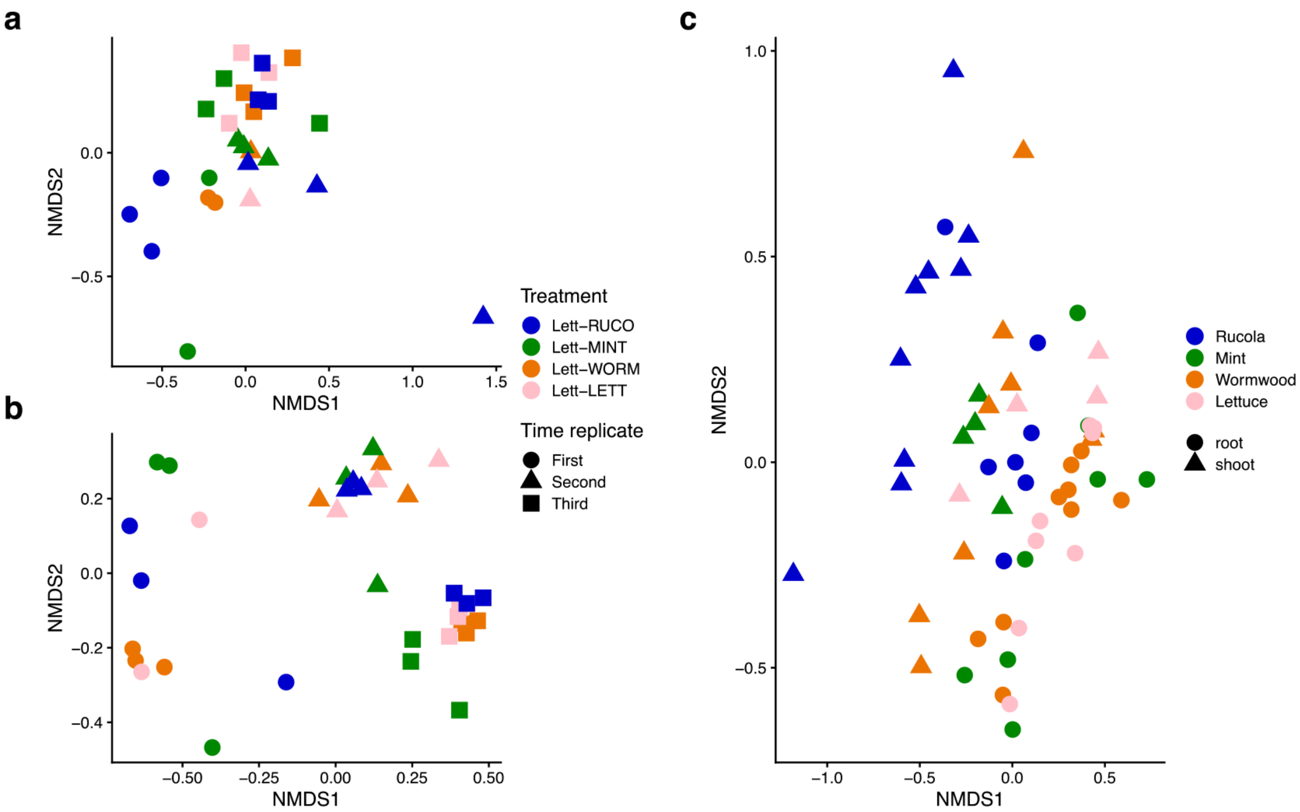

Fig. 2 Bacterial community in lettuce and companion plants in a decoupled aquaponic system. Non-metric multidimensional scaling (NMDS) plot of bacterial communities in A) lettuce shoot and B) lettuce root, when lettuce was grown with the companion species rucola (Lett-RUCO), spearmint (Lett-MINT), wormwood (Lett-WORM) or lettuce (Lett-LETT) in three time replicates for 30 days. Stress = 0.13. C) NMDS plot of the bacterial community in companion plant species roots and shoots. Stress = 0.166.

Figure 2

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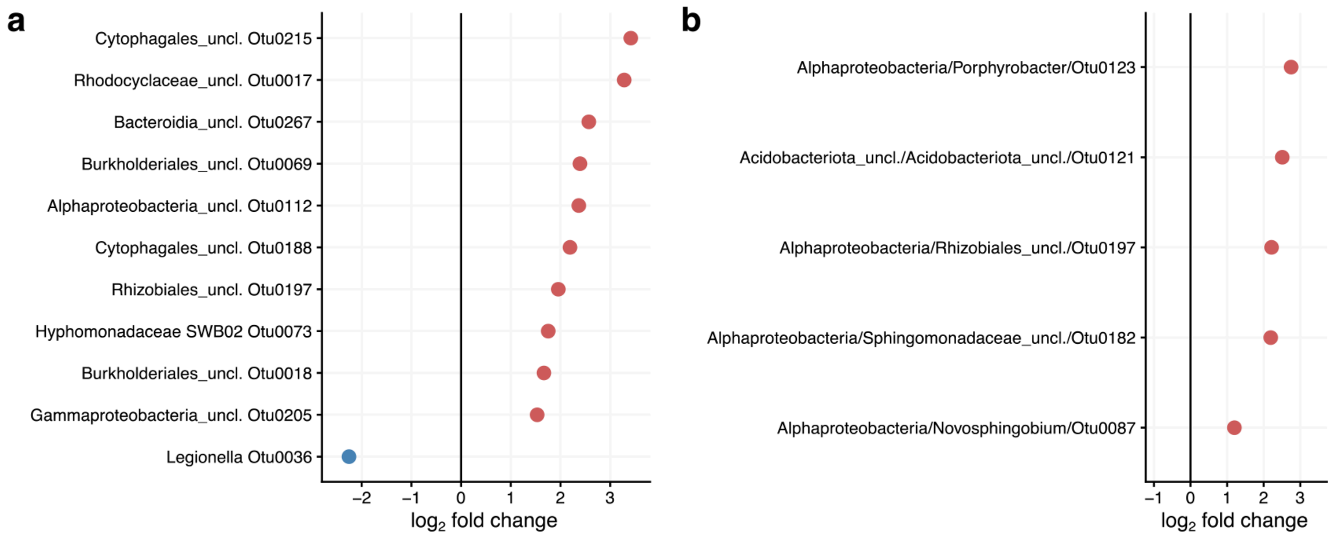

Fig. 3 Lettuce bacterial taxa promoted by spearmint and wormwood as companion plants. Differentially abundant bacterial operational taxonomic units (OTUs) in A) roots of lettuce grown with spearmint as the companion plant and B) lettuce grown with wormwood as the companion plant in a decoupled aquaponic system for 30 days. The OTUs shown were detected by at least two different differential abundance tools. Positive log$_2$ fold change values (red) from Deseq2 analysis indicate enrichment of OTUs with companion plant and negative values (blue) decrease of OTUs compared to lettuce grown alone. Taxonomic affiliations are shown at the genus (A) and class/genus (B) level. Uncl. denotes unclassified.

Figure 3

See image above for figure legend

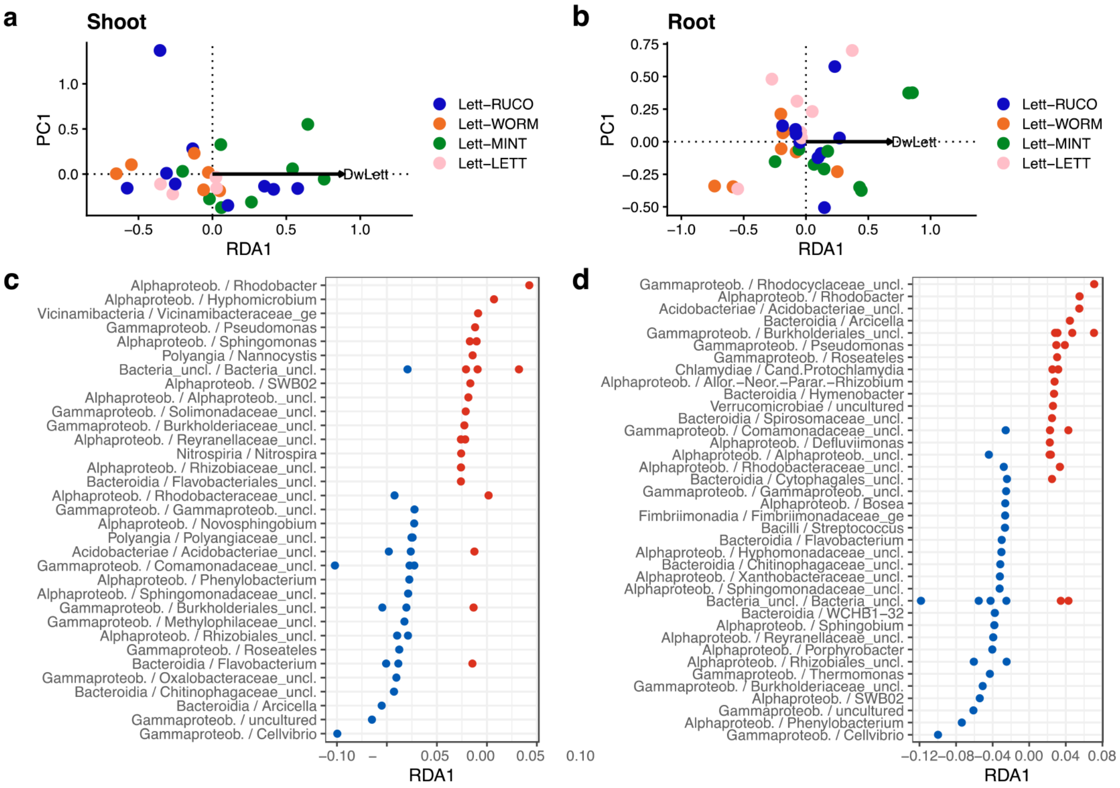

Fig. 4 Bacterial taxa associated with lettuce growth. Redundancy analysis (RDA) on A) lettuce shoot biomass effect on the bacterial community, B) lettuce root biomass effect on the bacterial community. C) Operational taxonomic units (OTUs) grouped at genus level that most strongly responded to lettuce shoot biomass and D) root biomass when lettuce was grown with its companions i.e. rucola (Lett-RUCO), spearmint (Lett-MINT), wormwood (Lett-WORM) and lettuce (Lett-LETT). Uncl denotes unclassified and DwLett denotes lettuce dry weight. Each dot represents an OTU where red indicates association with high biomass and blue association with low biomass. The plants were grown in a decoupled aquaponic system for 30 days.

Figure 4

See image above for figure legend

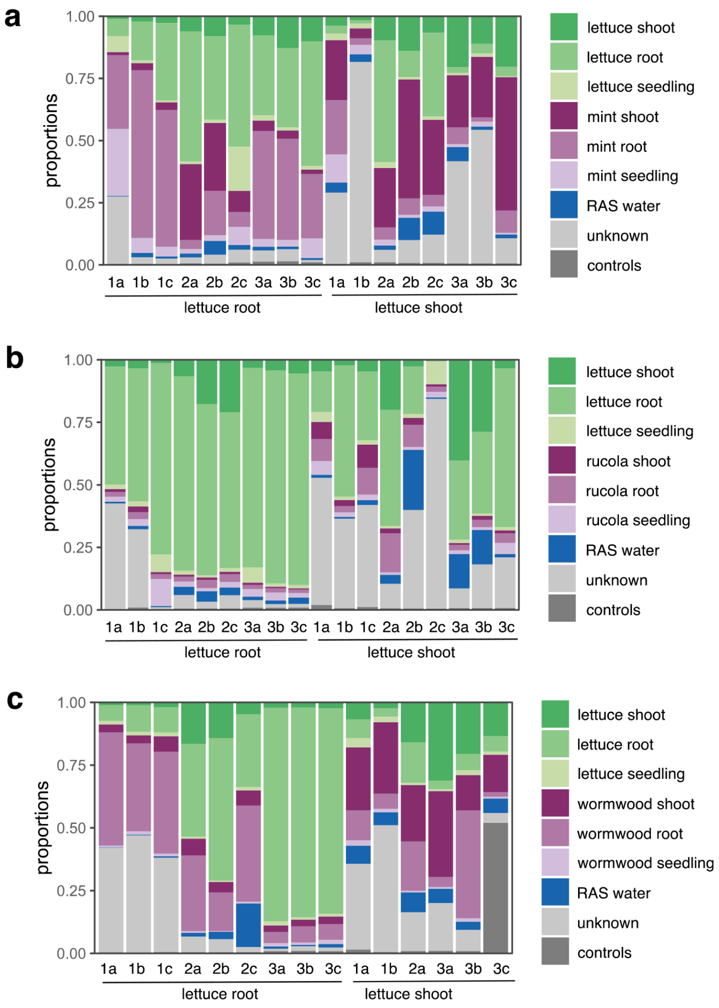

Fig. 5 Relative proportions of potential sources of bacteria in lettuce based on SourceTracker analysis. Lettuce was grown in a decoupled aquaponic system with A) spearmint, B) rucola, and C) wormwood. On the x-axis are the sink compartments where numbers 1-3 in sample codes refer to time replicates and letters a-c to replicate samples within the time replicates. The legend shows the bacterial sources where controls = negative controls from DNA extraction and PCR, unknown = the proportion of sequences from none of the previous sources.

Figure 5

See image above for figure legend

Supplementary Files

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