Pilot: Microbial diversity in agricultural systems and its effects on the human microbiome

An exploratory basis for follow-up research

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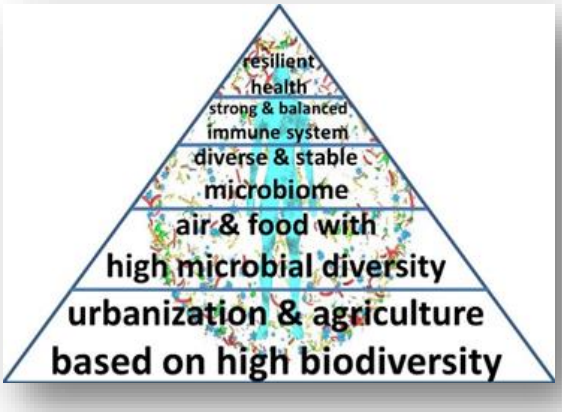

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1. Introduction

Microbiome and healthy plants and humans

The microbiome is of major importance to human and plant health (Banerjee and Van der Heijden 2023). The collection of microbes that an organism carries, is unique to every individual and can consist of many species comprising bacteria, fungi, yeasts and viruses. The microbiome contains species that are beneficial to the organism but can also contain harmful ones. For an organism to be healthy, the beneficial microorganism must outcompete the harmful ones.

For the human immune system it is essential to have intestines which are populated by a healthy gut microbiome (Tischer *et al.* 2022). Also for plants to be able to grow and prosper, a well-balanced soil/root microbiome is essential (Banerjee and Van der Heijden 2023, Brevik *et al.* 2020). In healthy ecosystems, soil organisms preserve or improve soil quality and structure, and decompose organic material as part of the nutrient cycling. In the rhizosphere -the soil surrounding the roots and influenced by plant root exudates - a healthy microbiome is required to provide the plant with necessary nutrients and to defend against pathogens. Plants actively recruit beneficial microorganisms in the rhizosphere (Berendsen *et al.* 2012). From a functional point of view one can make a comparison between the microbiomes in the humane intestines and the rhizosphere.

The crucial role of the microbiome in human health rapidly receives more and more attention (Soto-Giron *et al.* 2021). The interest in the role of the microbiome in the plant rhizosphere is growing, but is still mostly overlooked or marginalized in the current recommendations for preserving or stimulating soil health (Bender *et al*. 2016). Although the relation between soil and human gut microbiome is hypothesized (Blum *et al.* 2019), little to nothing substantial is known about the relation between the soil microbiome, plant microbiome and human health (Keijzer *et al*. 2020).

Organic farmers have long since recognized the importance of sustaining and stimulating a living healthy soil, with measures like the application of compost or manure, the use of cover crops, reduced or no tilling and extensive crop rotation. Various studies showed that the soil in organic production systems (e.g. in cereal, fruit and vegetable production) contain a greater microbial carbon biomass, more diversity and more microbial activity than conventional production systems (Widmer *et al*. 2007, Reeve *et al*. 2010, Gomiero *et al*. 2011, Hartmann *et al*. 2015, Lori *et al*. 2017, Martínez-García *et al*. 2018). In contrast, other researchers report on similar soil microbiomes between conventional and organic farming systems with similar soil structures and pH (Armalytė *et al*. 2019).

The influence of the management system on the microbiome was first studied in the Austrian project https://applebiome.com at the University of Graz. In an exploratory experiment they compared supermarket bought apples of organic and non-organic production systems. The *amount* of microbiome found was comparable, but the *species diversity* differed strongly between organic and conventional production. Inside the apples (especially on the seeds) more microbiome was found than on the outside of the apples (Wassermann *et al.* 2019). Differences in microbiome between organic and non-organic management were confirmed for fresh rocket salad grown under these two conditions by Mantegazza *et al.* (2023).

Research in harvested seeds of rapeseed (Brassica napus) showed that the farm environment in which the rapeseed was grown had a much larger effect on the diversity of the microbiome found than the genotype of the sowing seed used to produce the harvested seeds (Morales Moreira *et al*. 2021).

The question is whether different crop management systems result in and make use of different microbiomes in the soil, and subsequently lead to differences in microbiome in the harvested plant parts used for food. Generally, it is hypothesized that a healthy soil is the basis for a diverse and stable microbiome which results in a resilient health (see Figure 1):

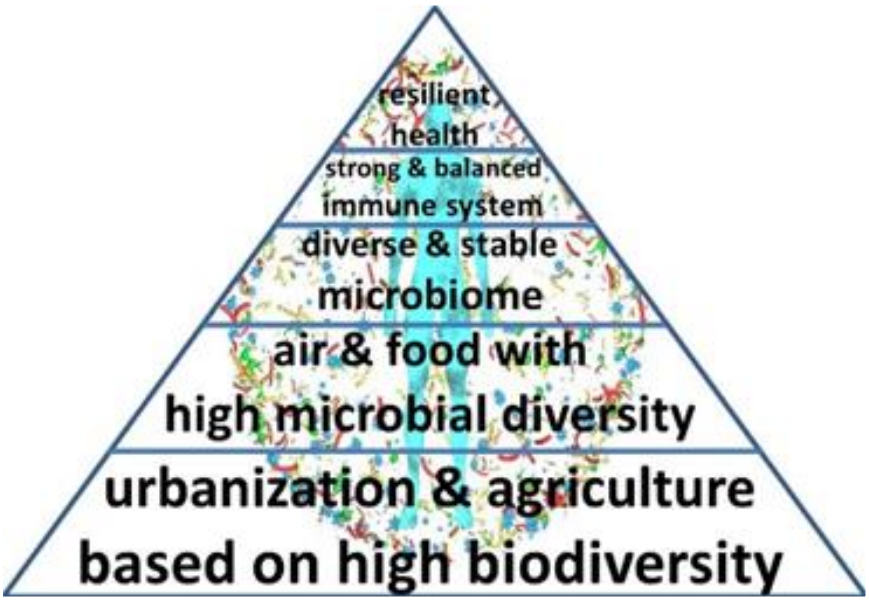


Figure 1. Hypothesized relation between a rich and diverse microbiome in agricultural production (e.g. soil) through food and gut microbiome leading to health and resilience in consumers of that agricultural produce (source: Bac2nature)

It is estimated that in the total human microbiome 100 billion cells of just bacteria are present. The gut microbiome is very dynamic: it refreshes itself every 7 days. There is a growing understanding of the relationship between the development of the gut microbiome during childhood and health in adulthood. An important function of the gut microbiome is the protection against pathogens. A relationship has been found between the composition of the gut microbiome and certain afflictions and diseases like e.g. intestinal disorders, asthma, obesity and type 2 diabetes (Frati *et al*. 2018, Sharma and Tripathi 2019, Aoun *et al*. 2020). It is still unclear how and why the microbiome differs between healthy people and people suffering from illness. Furthermore, there is still little knowledge about the variation of our gut microbiome over time; days, weeks, months and possibly years and to what extent this variation is influenced by our diet and possibly relates to, for example, the microbiome of our soils.

Vision

Research into microbiomes has gained momentum. Many relationships between microbiome and health are shown, but cause and effect are still mostly unclear.

In the coming years, we foresee an increasing interest into the influence of the microbiome in the soil, plants, animals and humans. These projects invariably focus on small bits in the entire food production chain. Understanding the holistic relationship between a healthy soil and human health could potentially provide multiple and synergistic leverage points to increase sustainability in land use and food production on one side, while simultaneously improving human health and combat certain non-communicable diseases resulting from poor diets on the other side.

So instead of solely focussing on one or two small steps we want to study the entire chain between soils, crops, food and human health. However, in order to do so we have to start with an exploratory research to get a first grip on this extensive and complex research area. This proposal outlines a first exploratory pilot for a broader future research agenda. Such a broader research agenda could be:

What interactions are there between soil, plant, animal and human microbiome?

- How is the relationship / interaction between the microbiome of soil, (unprocessed) crops, processed food, animal products and human health? What is the intermediate role of the plant between the microbiome of soil and humans?

How is the microbiome affected?

- What is the role and relationship between genetic and environmental factors in the construction, maintenance and recovery of the microbiome?
- Can the microbiome in the soil be affected in such a way that it increases soil resilience to diseases and pests and contributes to plant health and resilience? Can the soil microbiome then increase levels of nutrients beneficial for human health and secondary metabolites in crops?
- Can the gut microbiome be affected in such a way that it supports human health, or contributes to the recovery of diseases?

What are the threshold and target of diversity?

- What factors play a role in microbiome differences between humans and in age groups[4]. Is this a (slow) shift in industrial society or how does co-evolution work? Same for farm animals and intensive livestock farming.
- What type of diversity / composition is desirable and to what extent?

How can a gut microbiome (in humans and animals?) be repaired after antibiotic use?

- How is recovery of the gut microbiome (short and long term) possible after antibiotic use and subsequent: can the gut microbiome be preventively maintained in such a way that antibiotic use can be reduced (thus avoiding multi-resistance)?

In order to later arrive at this broader perspective, in this exploratory research we want to focus on the microbiome of tomatoes from organic and non-organic cultivation in order to study the potential differences in cultivation systems and subsequent effect on the microbiome in the intestinal tract. Based on the results from this exploratory research, we can then define further and more specific research questions and design appropriate experiments.

[4] Especially focused on the intestinal microbiota (wishes for diversity differ considerably here compared to e.g. skin or vaginal microbiome)

Hypotheses

Within this exploratory pilot we focus on three basic hypotheses we want to test:

1. Organic and non-organic cultivation method or management system lead to differences in microbiome of soil/substrate and in or on edible plant parts.
2. Microbiome in/on raw eaten vegetables reaches and influences the human gut microbiome.
3. Raw eaten vegetables with a different microbiome differ in their effect on the microbiome in the human intestine.

Based on the results of this research, more specific hypotheses and follow-up questions can be addressed in a broader consortium context.

Aim and scope of this pilot

With this exploratory research we want to test the hypotheses mentioned above, and hope to demonstrate differences between organic and conventional production systems expressed as differences in the microbiome in the soil/substrate, plant roots as in the edible plant parts.

Next we want to investigate the relation between consumed microbiome and gut microbiome. With that we hope to demonstrate a relation between the microbiome affected by cultivation system and – through the effect on the microbiome of consuming edible plant parts – on the microbiome in the human intestine.

If these relations are indeed found, the groundwork for further research into harnessing the microbiome into producing healthier food and research into food microbiome and human health effects, would be laid.

Specific research questions

- Does the microbiome contained in the seed lot used for sowing, relates to the microbiome found in the growth medium, plant roots and harvested edible plant parts?
- Can we find evidence for the "heritability" of the microbiome through seed generations by comparing the microbiome found in the seed in the harvested fruit to the microbiome present in the seed lot used for sowing? .
- Does crop management (organic vs conventional production) produce different microbiomes present in the same crop?
- Does the growth medium (organic soil versus Rockwool substrate) produce different microbiomes on the same crop in roots or harvested edible plant parts?
- Is the microbiome found in tomato fruit pulp and in the seeds identical?
- Do different microbiomes in tomato fruit pulp or seeds differ in their survival rate to the human gut microbiome?
- Can any similarities be found between the microbiome of edible plants parts and the already known species of the microbiome in the human gut?
- Can we relate found effects of food microbiome on the human gut microbiome to known beneficial health effects in literature?

2. Materials & Methods

In order to establish if there is a relationship between the microbiome on seed, soil, plant roots and the harvested edible plant parts, we start by establishing possible differences in microbiome of food which is consumed raw in relation to the crop management system, and to establish what part of that microbiome is viably transmitted to the gut microbiome and explore if this could have an effect on human health.

Tomato was the model crop for this pilot study as in the Netherlands tomatoes are usually consumed raw (Borgdroff-Rozeboom 2012), this to exclude food preparation effecting the microbiome. Moreover tomato offers the advantage that it can be separated into fruit pulp and seeds since initial studies in apple showed that the seeds contain the largest differences in microbiome (Wasserman et al. 2019). Besides we want to explore whether a certain microbiome can be stored into the seeds, which can influence the microbiome in the next growing season using these seeds for sowing, a so called 'seed heritability' or 'seed legacy' effect.

The project consists of three steps: 1) Tomato sample collection, 2) microbiome analysis, 3) TIM analysis. The steps are described in detail below.

Step 1. Tomato sample collection

For both conventional and organic production three growers were selected (six in total). At every production site we sampled two locations in the greenhouse (to reduce location effects in the greenhouse like shading or being near an outer wall where temperatures may be different). Samples from five individual plants per location/site in the greenhouse were pooled. Along with the tomatoes also the soil or hydroponic liquid was sampled, plus a leaf sample of the plants from which the tomatoes were harvested. As a result each grower provided nine samples (Figure 2). A sampling protocol can be found in Appendix 1.

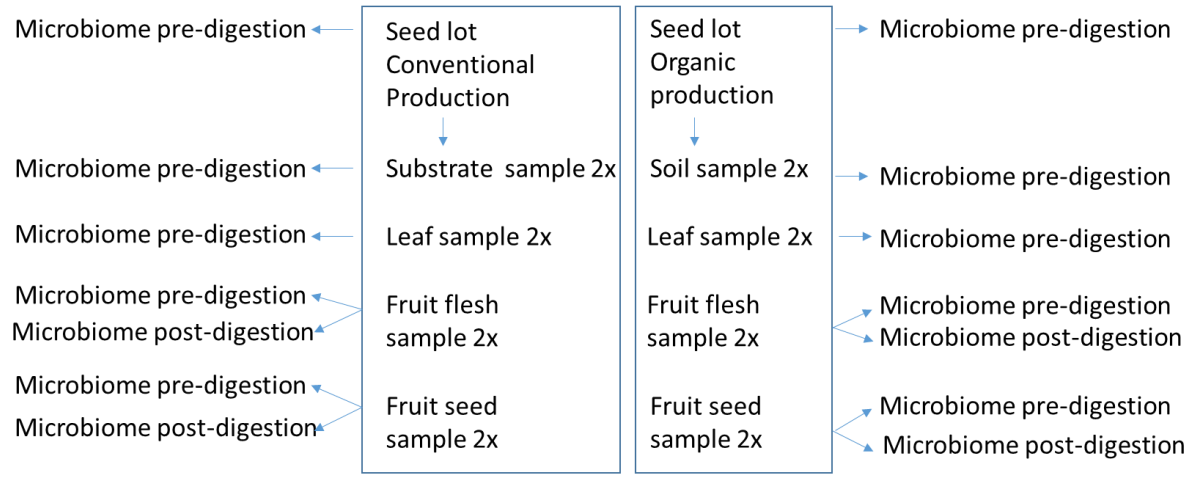


Figure 2. Microbiome sample overview

Step 2. Microbiome analysis

Microbiome analysis of fruits, leaf and growth medium (soil or hydrophonic) was outsourced to a commercial service provider BaseClear (https://www.baseclear.com/).

All qPCR reactions were performed in 384 well PCR plates (Thermo Fisher Scientific) sealed with MicroAmp Optical Adhesive Film (Thermo Fisher Scientific) using an Applied Biosystems QuantStudioTM 5 Real-Time PCR system (Thermo Fisher Scientific) with QuantStudioTM Design & Analysis software v1.4.2.

Each reaction for the total bacteria qPCR assay targeting the 16S rRNA gene was carried out in a total volume of 10 µl, with 5 µl ABsoluteTM Blue qPCR Mix, Low ROX (Thermo ScientificTM), 0.2 µl forward primer (5'-CGGTGAATACGTTCYCGG-3'; 10 µM), 0.2 µl reverse primer (5'-GGWTACCTTGTTACGACTT-3'; 10 µM), 0.1 µl probe (FAM-CTTGTACACACCGCCCGTC-BHQ1; 10 µM), 2 µl PCR grade water and 2.5 µl undiluted template DNA. A standard curve comprising 8 serial 10-fold dilutions of a synthetisized, cloned, linearized, and purified DNA of 192 bp, was generated from a work solution (0.1 ng/µl) that in turn was derived by 100 times diluting a stock solution (10 ng/µl). The PCR program started with a denaturation step at 95 °C for 15 minutes, followed by 40 cycles consisting of denaturation at 95 °C for 15 seconds, annealing and elongation at 52 °C for 1 minute (with data collection).

A positive control was performed alongside each separate amplification consisting of 2.5 µl of 0.1 ng/µl DNA (0.25 ng DNA added to a single reaction) that was derived from ZymoBIOMICS<U+2122> Microbial Community DNA Std. (D6306; Zymo Research). Negative template control (NTC) PCRs were performed alongside each separate amplification without addition of template.

Amplification data were exported from QuantStudio™ Design & Analysis software v1.4.2 followed by determining the target quantity per µl DNA preparations using the standard curves and calculation of the number of target per gram or ml of raw material using the formula below.

$$Quantity\ per\ unit\ material = \frac{Quantity * D_{DNA} * V_{DNA} * \frac{V_{lysis}}{V_{lysis.extraction}}}{M_{material}\ or\ V_{material}\ or\ N_{material}}$$

The samples that were analysed for microbiome:

- Seeds from the seed lot used for sowing the crop
- Substrate: soil or Rockwool plus the nutrient solution provided
- Plant leaves
- Harvested fruits: flesh/fruit pulp (before and after TIM)
- Harvested fruits: seeds (before and after TIM)

Molecular analyses was carried out by Maastricht University, focussing on the ribosomal 16S rRNA gene of bacteria and the ITS gene in fungi.

Via sequencing and database comparison the microbial genera were determined. The microbiome of the fruit pulp and fruit seeds were determined before the samples enter the TIM (pre-digestion) and after the TIM (post-digestion).

The surviving species detected after TIM were compared to species known to have a beneficial effect on the human health (probiotics). This will provide a first indication whether agricultural management systems differ in their potential to contribute to human health through the microbiome.

The microbiome diversity within the samples (alpha-diversity) is measured using the parameters: the Shannon index, the Faith's phylogenetic diversity index, and the Pielou's evenness metric (Xia 2023a). The Shannon index is a weighted diversity assessment. Both the number of species and how often they occur are included in the calculation. The maximum value is reached when each measured species is equally present, so a higher score means that many measured species have approximately the same number of species. Faith's phylogenetic diversity (PD) (Faith 1992) is defined as the sum of the branch lengths of a phylogenetic tree connecting all species in the target assemblage. Pielou's evenness provides information about the equity in species abundance in each sample, in other words are some species dominating others or do all species have quite the same abundances.

The microbiome diversity between samples (beta-diversity) is measured using Jaccard dissimilarity (Xia 2023b). The Jaccard index is a similarity coefficient which does not take into account the abundance of the taxa but rather relies on presence or absence of the taxa (i.e. the composition of the microbiome). The Jaccard dissimilarity matrix is used for a principal coordinates analysis (Lu *et al.* 2021). Taxa barplots of all the samples are provided.

Step 3. TIM analyses

An artificial gastro-intestinal tract model (TIM) will be used to establish which part of the microbiome measured in unprocessed tomato samples survives passage through the human stomach and reaches the intestines, where it can potentially contribute to human health.

TIM is the TNO *in vitro* model of the GI tract (developed by TNO, implemented by Maastricht University)(Cordonnier *et al.* 2015, Uriot *et al.* 2016). The model mimics human (or animal) digestion and fermentation, with dynamic changes of the physiological parameters, such as pH in the gastric compartment, and concentrations of bile and pancreatic enzymes in the small intestine. The colon (or large intestinal) model, is inoculated with a dense active microbiota of fecal origin, which contains all of the hundreds of microbial species that are normally present in the human gut microbiota.

The gastro-intestinal TIM-1 system (TNO, Zeist, The Netherlands) is a multi-compartmental, dynamic, computer-controlled model that simulates the upper human gastro-intestinal tract (Table 1). TIM-1 consists of four successive compartments simulating the conditions found in the stomach and the three segments of the small intestine in humans, i.e., the duodenum, jejunum, and ileum. The main parameters of human digestion, such as pH, body temperature, peristaltic mixing and transport, gastric, biliary and pancreatic secretions, and passive absorption of small molecules and water, are reproduced as accurately as possible. Briefly, each compartment is composed of glass units with a flexible inner membrane. Peristaltic mixing and body temperature are achieved by pumping water at 37 °C into the space between the glass jacket and the flexible wall at regular intervals.

Chyme transport through the TIM-1 is regulated by the peristaltic valves that connect the successive compartments. The volume in each compartment is monitored by a (pressure) sensor, and pH is computer-monitored and continuously controlled by

adding either HCl (gastric compartment) or $NaHCO_3$ (intestinal compartments). Simulated gastric, biliary and pancreatic secretions are introduced into the corresponding compartments by computer-controlled pumps. Water and products of digestion are removed from the jejunal and ileal compartments by pumping dialysis liquid through hollow fiber membranes (SF 90G, Nipro, Osaka, Japan, with a molecular mass cut-off value of 10 kDa). Before each experiment, the system is washed with detergent, rinsed with water and decontaminated rinsing with ethanol.

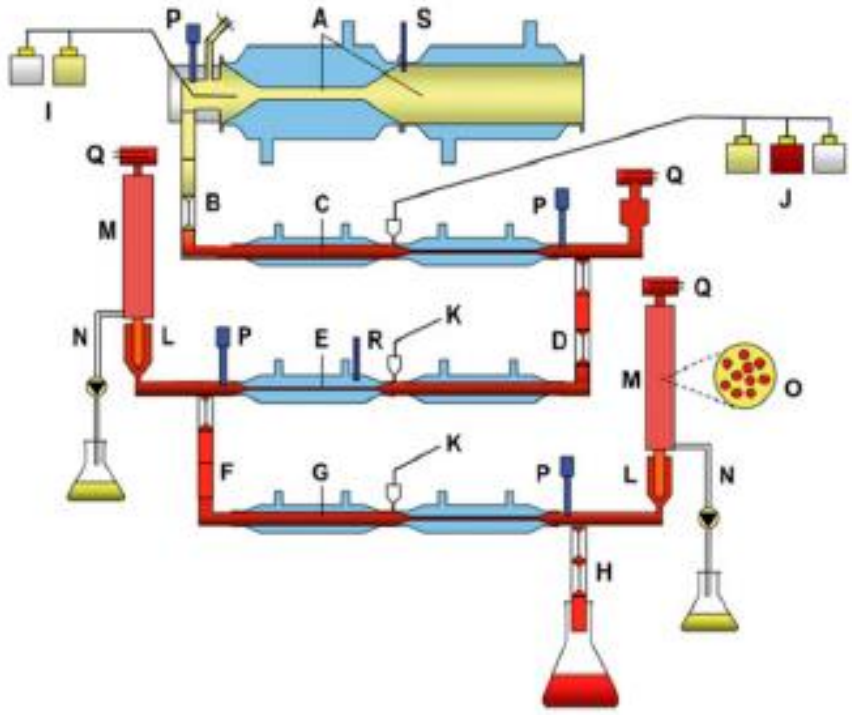


Figure 3. Schematic representation of TIM-1 from Cordonnier 2015: A: gastric compartment; B: pyloric sphincter; C: duodenal compartment; D: peristaltic valves; E: jejunal compartment; F: peristaltic valves; G: ileal compartment; H: ileal-cecal valve; I: gastric secretion (lipase, pepsin); J: duodenal secretion (bile, pancreatic juice, electrolytes); K: bicarbonate secretion; L: pre-filter; M: filtration system; N: filtrate with bio-accessible fraction; O: hollow fiber system (cross section); P: pH electrodes; Q: level sensors; R: temperature sensors; S: pressure sensor.

3. Results

Tomato sample collection

The choice to work with tomatoes proved to be difficult for collecting samples for two reasons: 1) non-organic tomato production is extremely walled-off to avoid introduction of a vast array of viruses and viroids, so finding growers who wanted to contribute to this pilot took an unforeseen large effort, and 2) tracking down seed lots used to raise tomato plants from or plant lots raised form the same seed lot proved to be prohibited by Dutch privacy legislation.

In March 2022 we posted an online call on LinkedIN and social media for non-organic tomato growers. Fortunately, the handful of organic tomato growers in the Netherlands are known to us, and we made arrangements with two of them. In April through July 2022 we contacted lots of non-organic tomato growers, resulting in two non-organic growers (next to the two organic) in July 2022, when we started with the collection of the tomato fruit/leaf/soil&hydroponic samples. Collected samples were brought the same day to Maastricht University campus site at Venlo for storage at -80 °C for later processing.

Tomato growers buy their plants grafted on rootstocks from specialized growers. Tracking down seed lots used and which variety is sold to which grower is confidential information which was not available to us. As a result we didn't succeed to take samples of the same variety. Varieties and root stocks sampled are indicated in table 1. Seed lots used to raise the tomato plants from were also not known and thus unavailable to sample, meaning the relation between microbiome in the initial seed and microbiome in resulting plants and tomato fruits could also not be investigated.

Table 1: Tomato varieties sampled

Grower	Management	Tomato variety	Tomato root stock
ENZA demo site (Pijnacker)	Non-organic hydroponic	(experimental hybrid)	Maxifort
Van der Knaap (Honselersdijk)	Non-organic hydroponic	Completon	Estamino
BioVerbeek (Velden)	Organic soil	Brioso	Fortamino
De Lepelaar (Sint Maarten)	Organic soil	Annamay F1	Maxifort

Samples collected are specified in table 2. Detailed description of the tomato crops sampled and growth conditions are described in Appendix 2.

Table 2: Samples collected for microbiome analysis

Grower designation	Growth management	Sample type	growth condition (sunny side/ shady side)
ENZA demo	non-organic	Fruit	sun
ENZA demo	non-organic	Fruit	shadow
ENZA demo	non-organic	Leaf	sun
ENZA demo	non-organic	Leaf	shadow
ENZA demo	non-organic	hydroponic fluid	
Van der Knaap	non-organic	Fruit	sun
Van der Knaap	non-organic	Fruit	shadow
Van der Knaap	non-organic	Leaf	sun
Van der Knaap	non-organic	Leaf	shadow
Van der Knaap	non-organic	hydroponic fluid	
BioVerbeek	Organic	Fruit	sun
BioVerbeek	Organic	Fruit	shadow
BioVerbeek	Organic	Leaf	sun
BioVerbeek	Organic	Leaf	shadow
BioVerbeek	Organic	greenhouse soil	
De Lepelaar	Organic	Fruit	sun
De Lepelaar	Organic	Fruit	shadow
De Lepelaar	Organic	Leaf	sun
De Lepelaar	Organic	Leaf	shadow
De Lepelaar	Organic	greenhouse soil	

Diversity indices

Measurement of diversity of species is one of the most important characteristics of phytosociology, e.g. the study of communities based on species composition and quantitative community structure (Thukral 2017). Diversity measurements can be performed at three levels, alpha, beta and gamma diversity (Whittaker 1976). Alpha diversity is the measurement of diversity within a habitat or intra-community diversity, in our case in each of the tomato fruit and leaves samples. Beta diversity is the measurement of diversity between habitats, in our case between tomato growers. Gamma diversity encompasses diversity at the landscape level.

Alpha diversity is the most widely used component in the characterization of communities. It generally has two components, species richness and evenness. Species richness concerns the number of individuals per unit area or sample, and species content of the area. These are also known as variety indices and are higher for species rich communities. Evenness indices indicate the relative abundance of different species of a community in terms of their evenness of distribution. A community in which the species have equal number of individuals of different species will have a higher evenness index. A community dominated by one species will have a low evenness index.

Microbiome analysis on samples of tomato fruits, leaves and growing substrate

Sequencing was performed by BaseClear, an extrenal service provider. BaseClear stated that with their method, chloroplast or mitochondrial DNA would not be picked up. However, for the tomato fruit samples, 93.5% to 99.6% of the sequences in the samples originated from these organelles. For the tomato leaves this was 74.7 to 99.7%. For the substrates samples (soil and hydroponic water) this was 0 to 1.4%. The chloroplast, tomato and mitochondrial sequences were filtered out of the dataset and the remainder of the sequences was used to do the analyses.

Tomatoes fruits and leaves were sampled separately from the sun-side and the shadow-side of plants. Since the microbial composition did not differ between the sun-side and shadow-side, the results described are the average of the two.

Microbiome diversity within samples (alpha-diversity)

Microbiome diversity within the samples (alpha-diversity) was assessed using the Shannon index, the Faith's phylogenetic diversity (PD) index and the Pielou's evenness index.

Bacteria on tomato fruits

For bacteria on tomato fruits, the Shannon index was not significantly different between growers (data not shown). A trend for a higher Shannon index for organic tomato fruits compared to non-organic tomato fruits was found (p=0.074; Figure 4A).

Similarly, the Faith's phylogenetic diversity (PD) index was not significantly different between growers (data not shown), but was significantly higher in organic tomato fruits compared to non-organic tomato fruits (p=0.016; Figure 4C).

For the Pielou's evenness metric, the value was significantly higher for Enza tomatoes compared to the other three growers (p=0.042 for all three comparisons; Figure 5A). Organic tomatoes had a significantly lower evenness index than non-organic tomatoes (p=0.046; Figure 4B).

Lastly, for observed taxa, Enza tomatoes showed a trend for lower number of features compared to the other three growers (p=0.06 for Bioverbeek and van der Knaap; p=0.084 for De Lepelaar; Figure 5B).

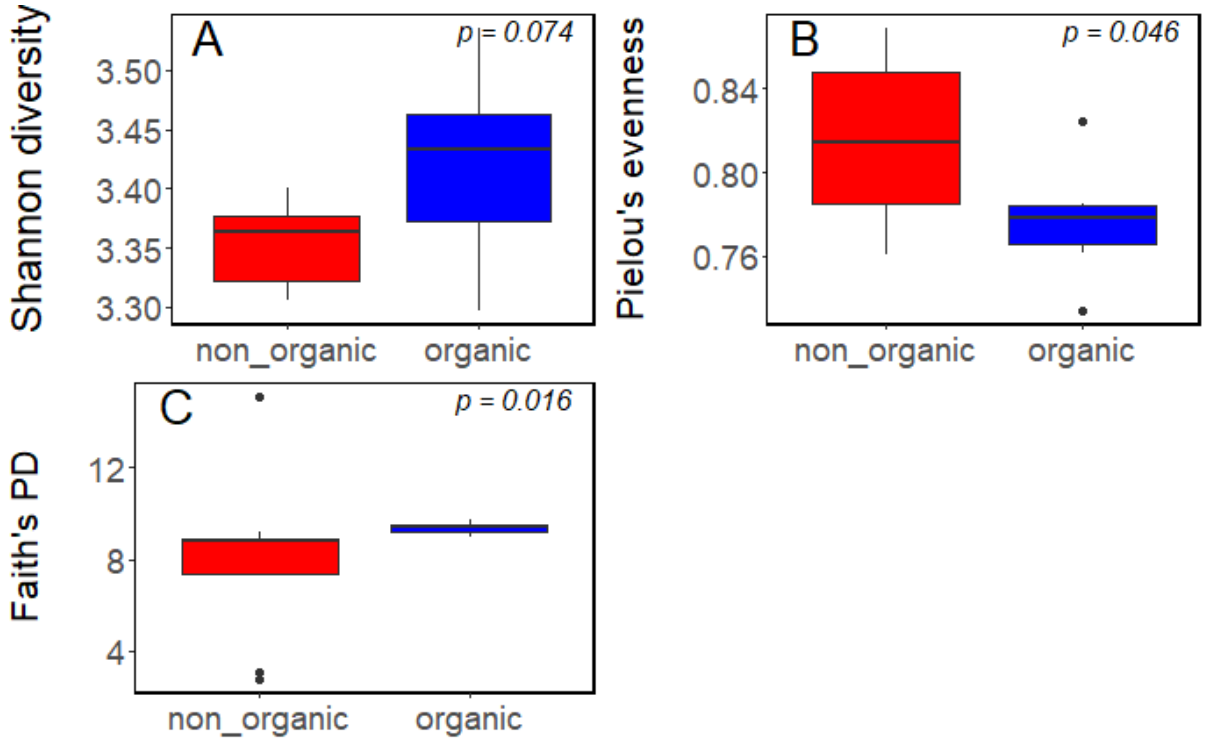


Figure 4. Alpha-diversity of bacteria within the organic and non-organic tomato fruits . A: trend for Shannon diversity; B: Pielou's evenness; C: Faith's phylogenetic diversity.

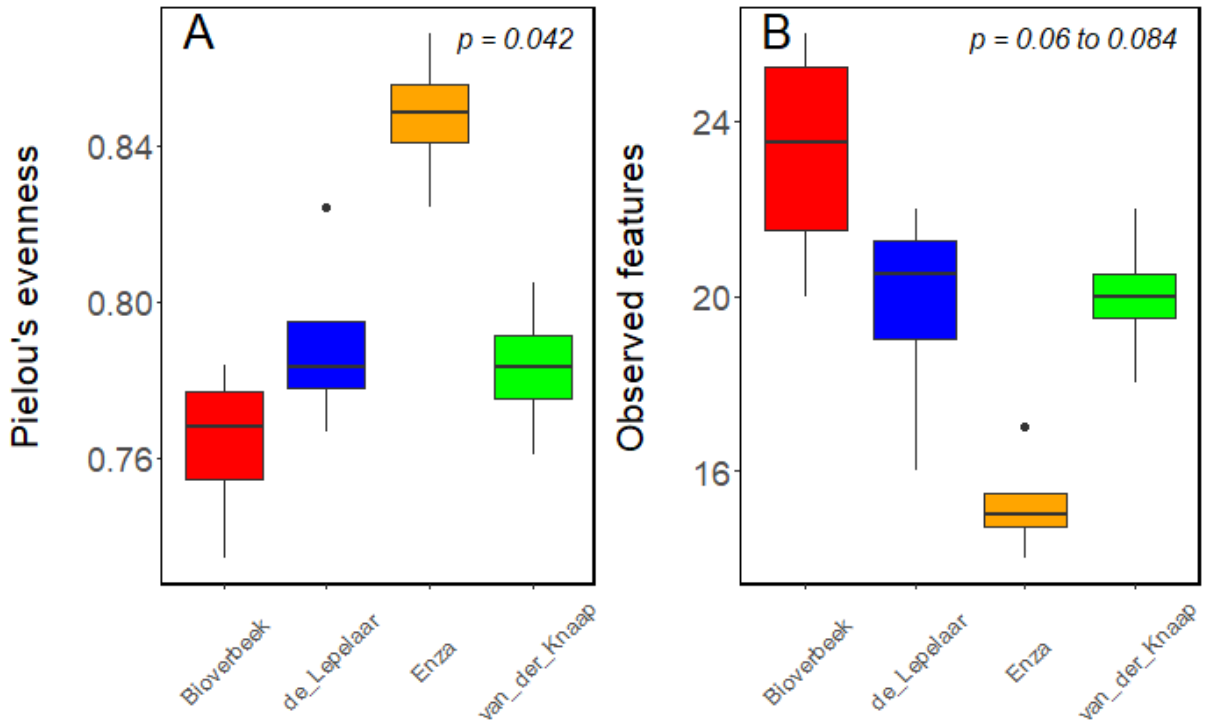


Figure 5. Alpha-diversity of bacteria within the tomato fruits divided by grower. A: Pielou's evenness; B: observed features.

Bacteria on tomato leaves

For bacteria on tomato leaves there were no significant differences in Shannon index, despite a much higher index for Bioverbeek, but this did not reach a trend (q>0.1).

There were no significant differences in Faith's PD index between growers, despite a much lower index for Enza (data not shown). However, the PD index was significantly higher for organic tomato leaves (q=0.025; Figure 6B).

There was a trend for a higher Pielou's evenness for Enza samples (as for tomato fruits; q=0.096 for all 3 comparisons), and therefore a trend in higher Pielou's evenness for non-organic samples (q=0.078; Figure 6A).

Lastly, there was a significant difference in observed features, being significantly higher for organic leaves (q=0.010; Figure 6C), primarily due to a low index for Enza.

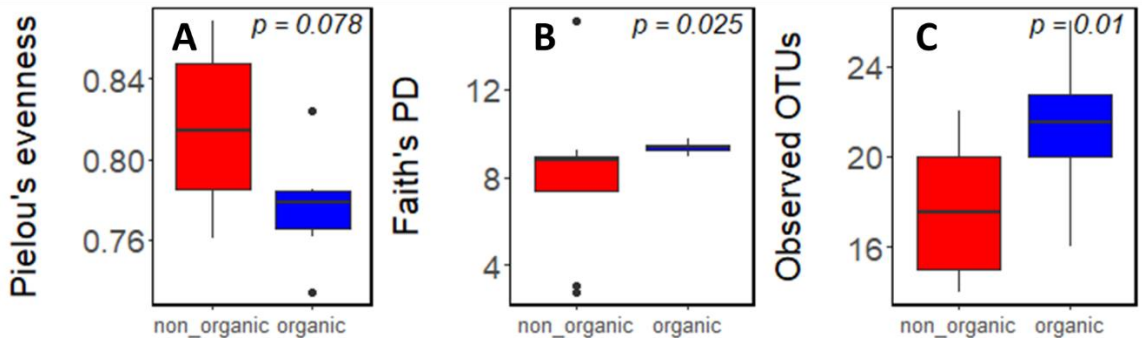


Figure 6. Alpha-diversity of bacteria within the leaves of organic and non-organic grown plants. A: Pielou's evenness; B: Faith's phylogenetic diversity; C: observed features.

Fungi on tomato leaves

A significant difference in the observed features of fungi on tomato leaves was found between organic and non-organic grown plants (q=0.027 Figure 7B).

There was a trend for a higher Faith's PD in organic grown tomato leaves compared to non-organic grown tomato leaves (q=0.065; Figure 7A). Differences between growers were not significant.

Only 7 fungal taxa could be classified beyond the fungal kingdom, the remaining being characterized as "Fungi" at the kingdom level. Unfortunately, this unclassified "Fungi" taxon took up 53%-100% of the relative abundance (see also below in taxa barplots) and led to the fact that there was little discriminative power between samples. In fact, 6 of the samples had this uncharacterized taxon as the only taxon. The other samples ranged from 3 to 6 taxa. Therefore, no conclusions can be drawn from this data.

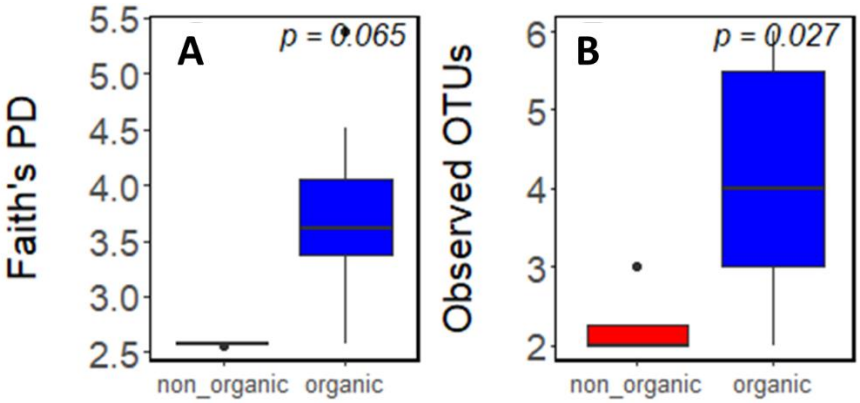


Figure 7. Alpha-diversity of fungi within the leaves of organic and non-organic grown plants. A: Faith's phylogenetic diversity; B: observed features

Microbiome diversity between samples (beta-diversity)

Bacteria on tomato fruits

Figure 8B shows differences in Jaccard dissimilarity between the growers (PERMANOVA *q*-value=0.031 for all individual comparisons; Figure 8B). The samples from each of the growers cluster together, indicating more similarity between the samples from a grower than between the growers. The samples from Enza (non-organic) and De Lepelaar (organic) are from the same rootstock 'Maxifort' and closer together than the samples from BioVerbeek and van der Knaap. This suggests that the differences between the growers are large and not necessarily related to organic or non-organic management styles but e.g. also due to effects of the rootstock used. However, a significant difference was found between organic and non-organic tomato fruits (PERMANOVA *q*-value=0.001; Figure 8A).

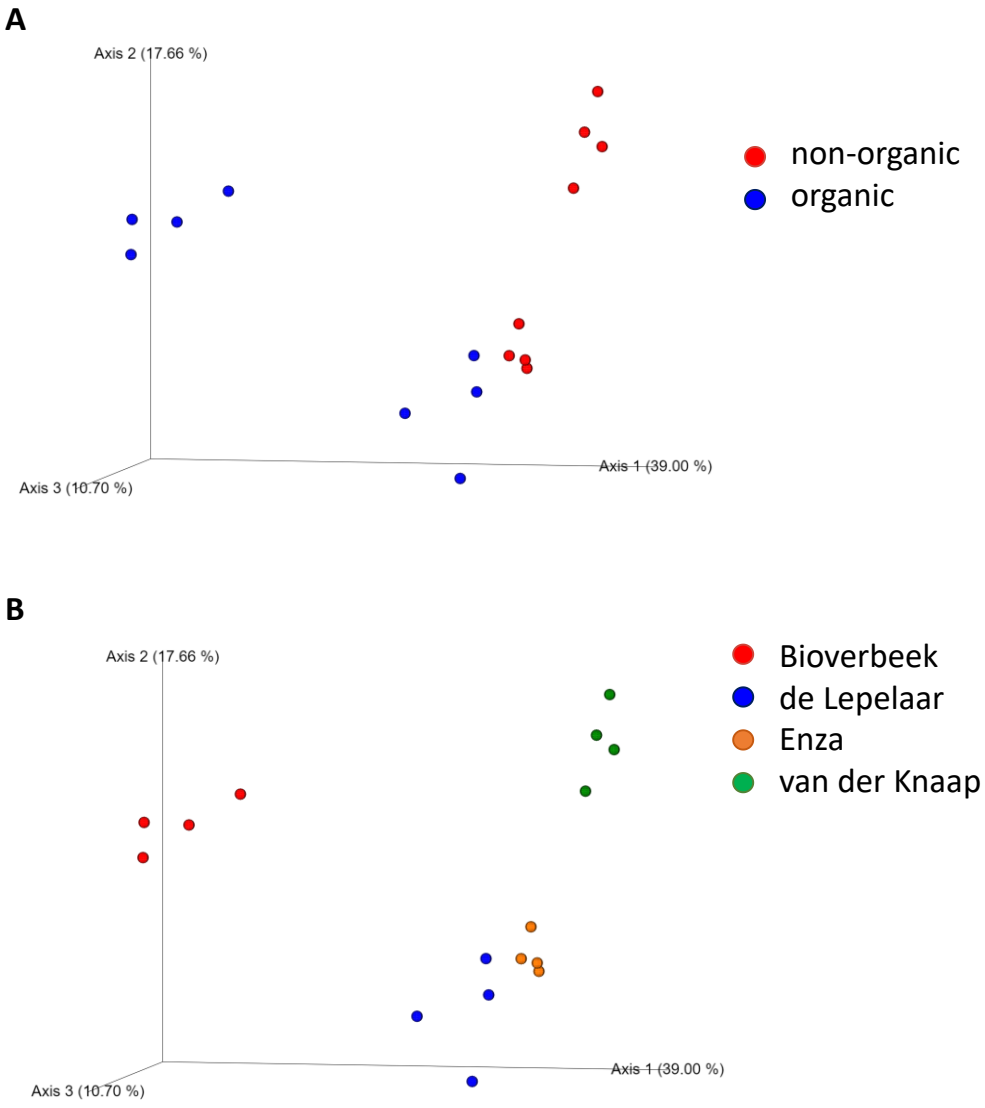


Figure 8. Beta-diversity of bacteria within the tomato fruits, indicated as Jaccard dissimilarity in a principal coordinate analysis. A: labelled organic versus non-organic; B: labelled for different growers.

Besides an overall community difference (beta-diversity) there also was a difference in some of the taxa in the samples. Two taxa (*Bacillus* [Figure 9A and C] and a taxon that could not be assigned by the bioinformatic pipeline, indicated as Unassigned [Figure 9B and D]) were significantly different between organic and non-organic tomatoes. The presence of *Bacillus* in the tomatoes of Bioverbeek is responsible for large differences between organic and non-organic growers, because *Bacillus* was absent in both organic growers and very low in De Lepelaar. The unidentified taxon (Unassigned) was also significantly different between organic and non-organic growers (Figure 9B) with large differences between individual growers (Figure 9D). It is therefore unclear whether the differences found can be ascribed to organic or non-organic growing conditions or other differences between the 4 farms.

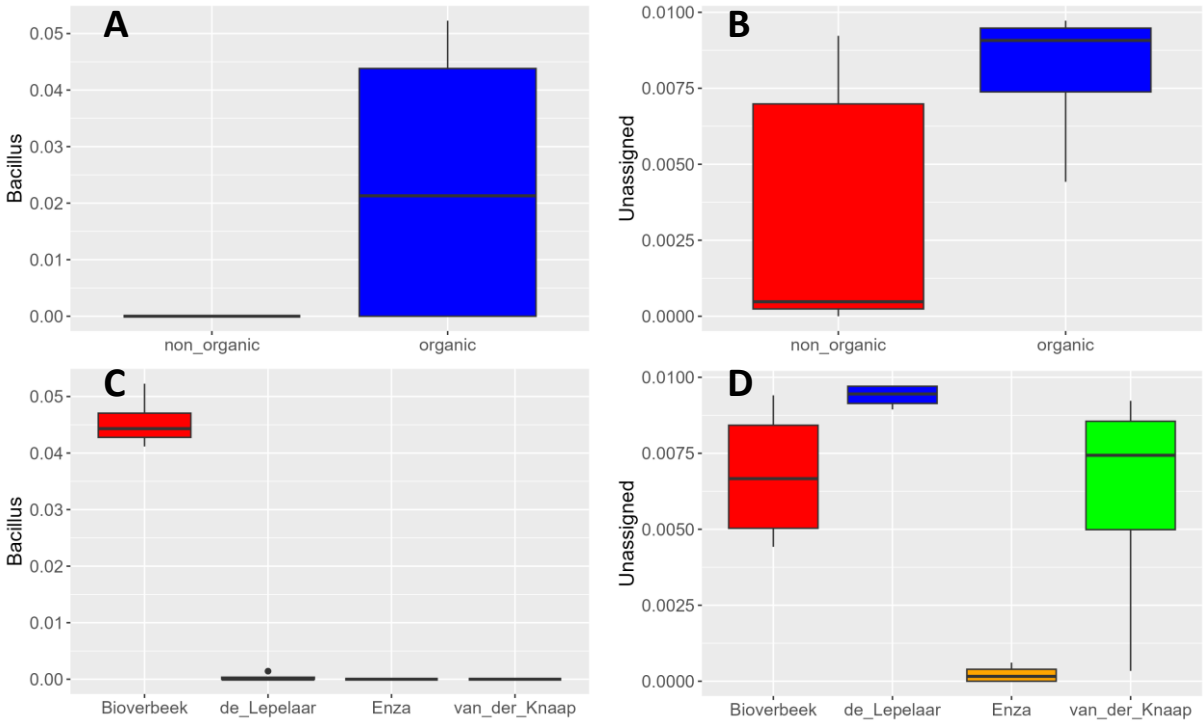


Figure 9. Box-plots of taxa within the tomatoes that are significantly different between organic and non-organic tomatoes. A and C: Bacillus (absent in non-organic tomatoes and two growers); B and D: an unassigned taxon; A and B: plotted as difference between organic and non-organic tomatoes; C and D: plotted as difference between growers.

Bacteria on tomato leaves

For the tomato leaves there also was a significant difference in bacterial composition with respect to overall beta-diversity for organic versus non-organic leaves (q=0.004: Figure 10A). This was not driven by a specific taxon, as none of the taxa was significantly different between organic or non-organic leaves.

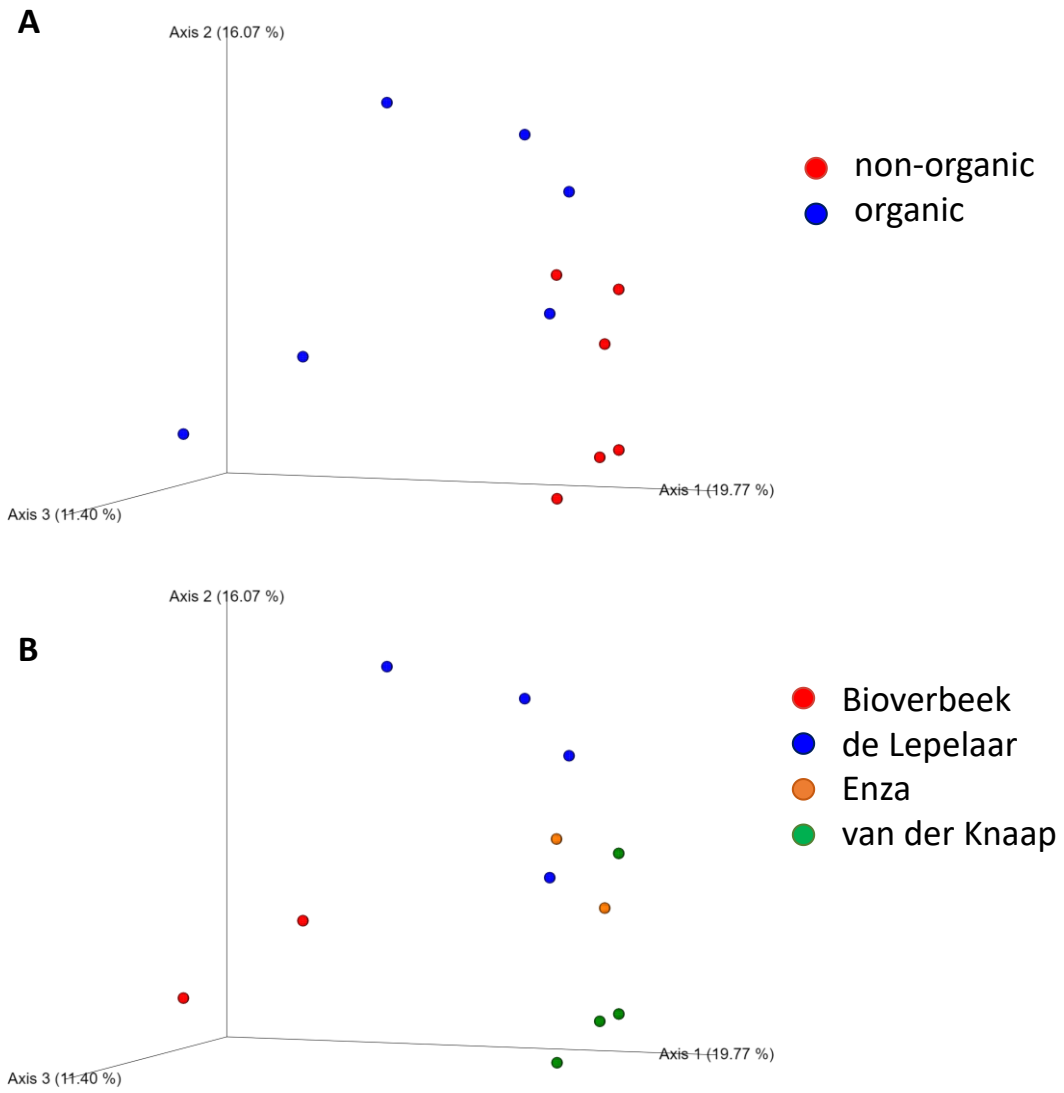


Figure 10. Beta-diversity of bacteria within the tomato leaves, indicated as Jaccard dissimilarity is a principal coordinate analysis. A: plotted as organic versus non-organic; B: plotted as the different growers.

Three taxa showed differences between growers (Figure 11), primarily because they were present in leaves from Bioverbeek and not in those of the other growers.

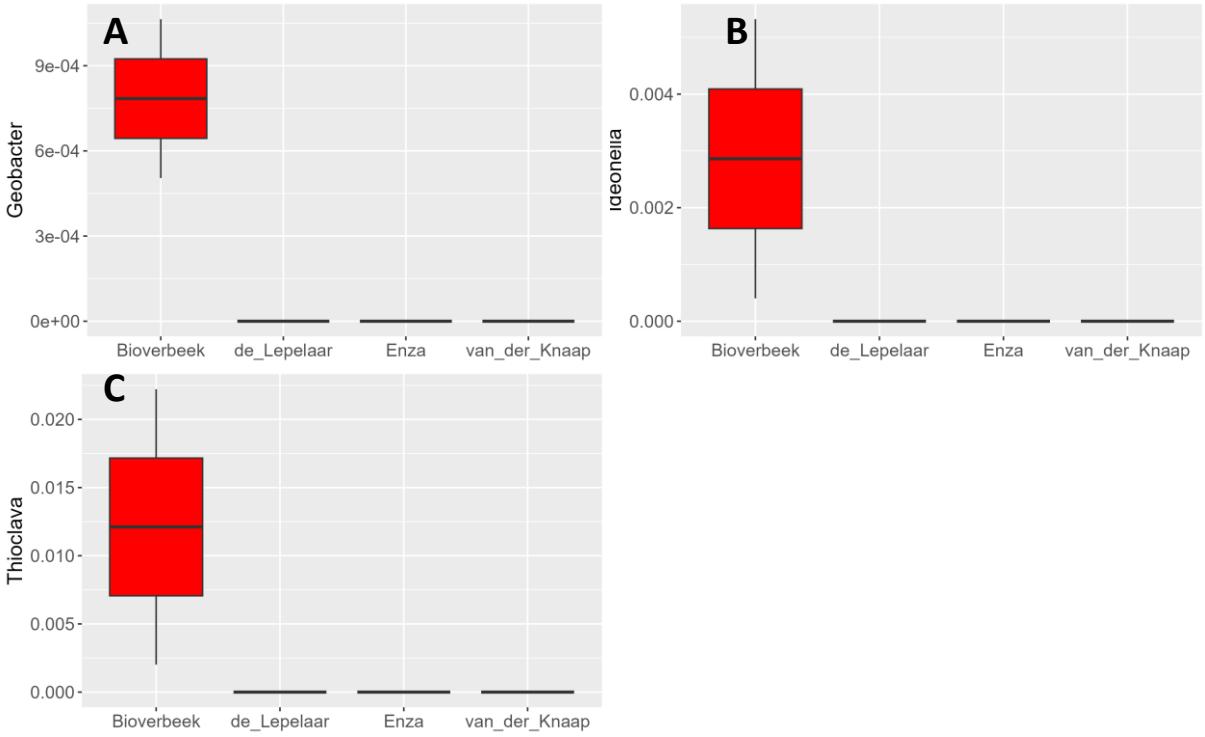


Figure 11. Box-plots of bacterial taxa within the leaves that are significantly different between growers.

Fungi on tomato leaves

For the tomato leaves there was no difference in beta-diversity for the fungal community between organic and non-organic plants (q=0.104; Figure 5). However, as mentioned in the section on alpha-diversity, there were only 7 taxa that could be classified beyond the fungal kingdom. The organically grown plants showed the highest number of different taxa on the leaves. An unidentified genus within the order "Entylomatales" was significantly different (q=0.048; not shown) between growers (due to its exclusive presence in De Lepelaar leaves; not shown), and because of this there was a trend for this taxon to be different between organic and non-organic leaves (q=0.081; not shown). The relevance of this is unclear, with so little different taxa in the dataset and a relatively small sample size.

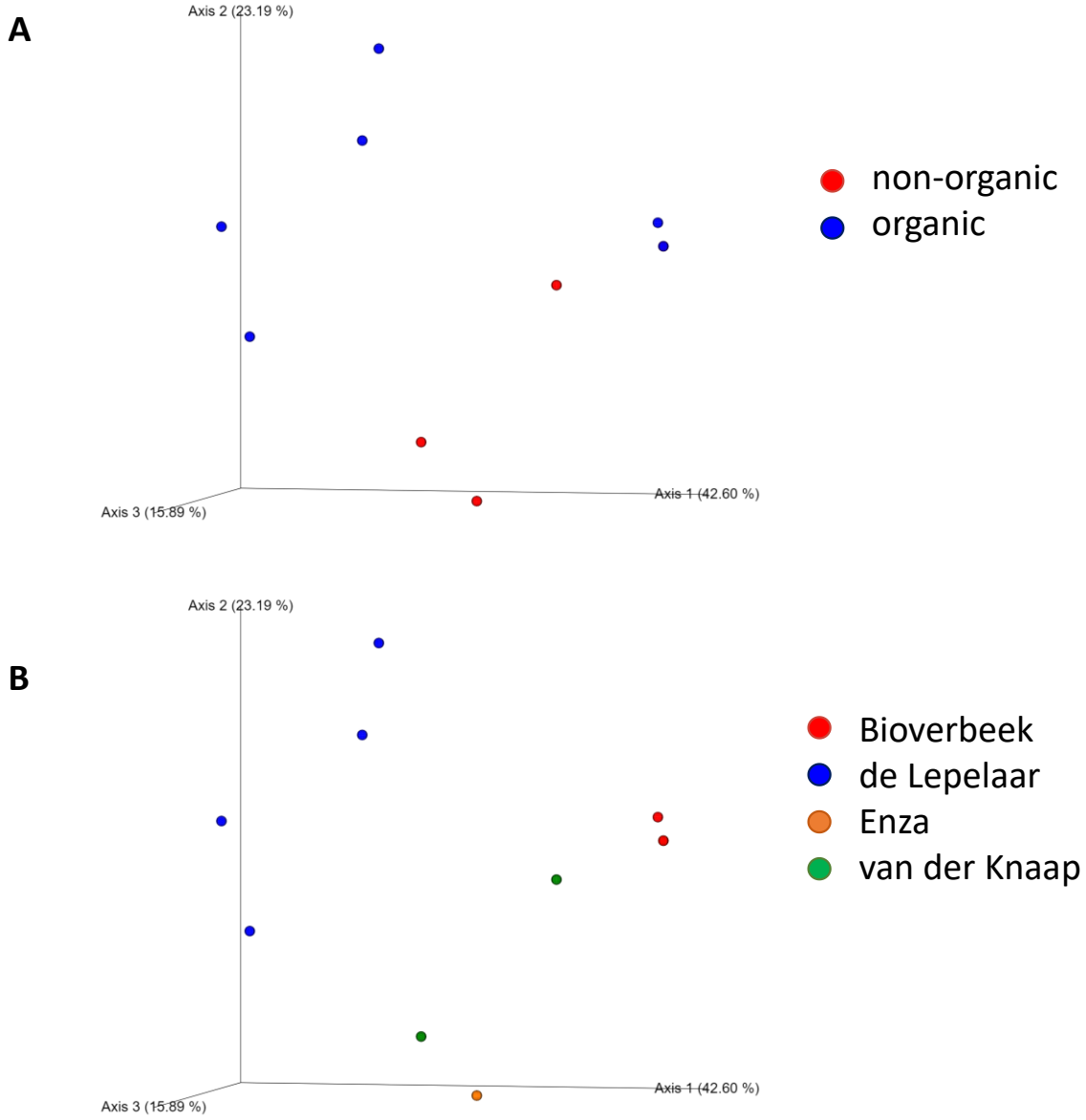


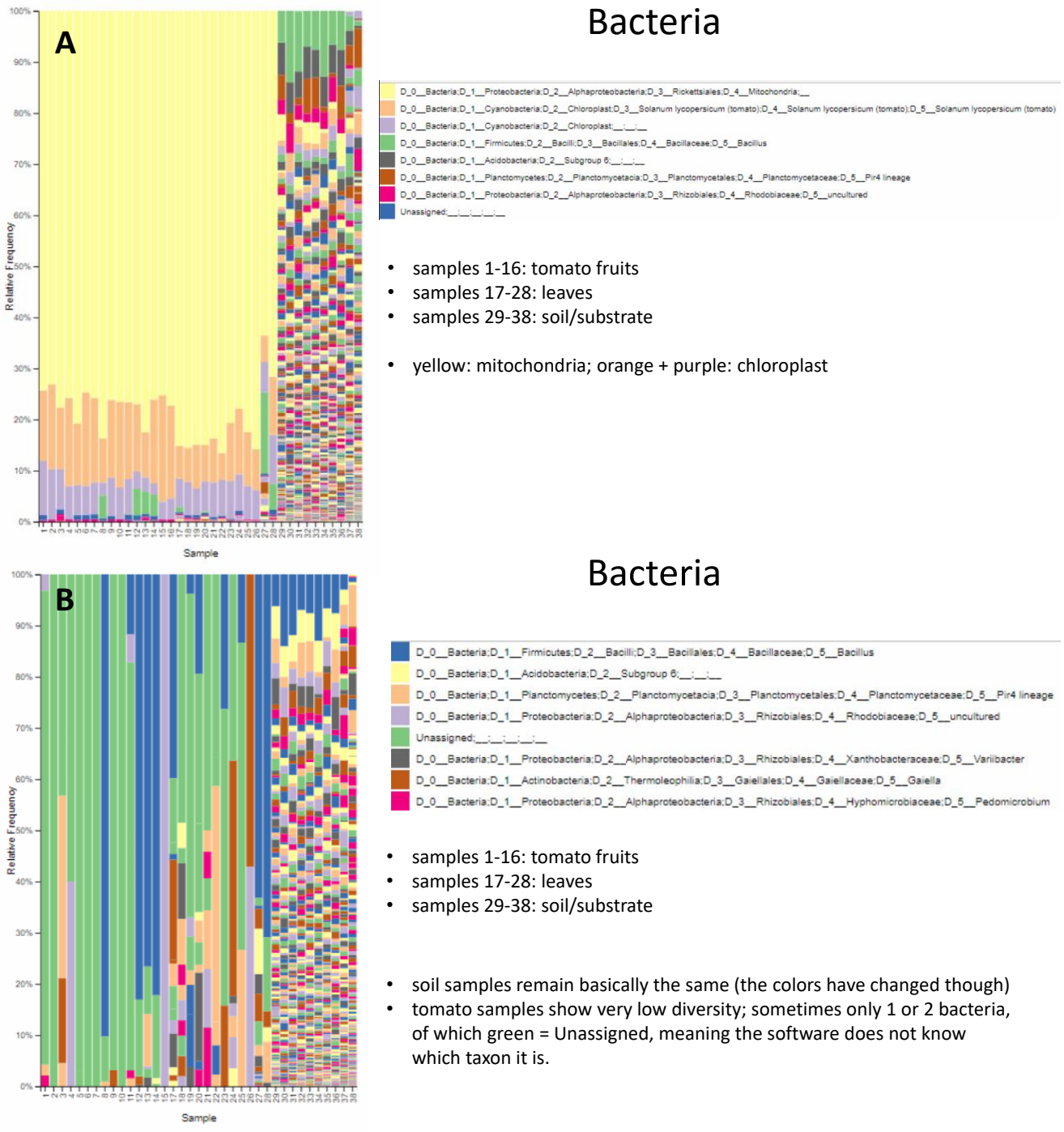
Figure 12. Beta-diversity of fungi within the leaves, indicated as Jaccard dissimilarity is a principal coordinate analysis. A: plotted as organic versus non-organic; B: plotted as the different growers.

Fungi on tomato fruits

For the tomato fruits, the number of assigned fungal taxa was too low to be representative (data is not shown).

Taxa barplots

Growing substrate was also evaluated. The taxa-barplot of the substrates is provided for bacteria (with and without the chloroplast and mitochondrial sequences; Figure 13A and B, respectively) and for fungi (Figure 14).



- samples 1-16: tomato fruits
- samples 17-28: leaves
- samples 29-38: soil/substrate

- yellow: mitochondria; orange + purple: chloroplast

- samples 1-16: tomato fruits
- samples 17-28: leaves
- samples 29-38: soil/substrate

- soil samples remain basically the same (the colors have changed though)
- tomato samples show very low diversity; sometimes only 1 or 2 bacteria, of which green = Unassigned, meaning the software does not know which taxon it is.

Figure 13. Taxa-barplots of the individual bacteria samples. A and B: bacteria, with chloroplast and mitochondrial sequences (A) and after filtering out these sequences (B); Samples 1 -16 are from tomato fruit; samples 17 to 28 from tomato leaves, samples 29 to 36 soil; samples 37 and 38 hydroponic fluid and coconut substrate.

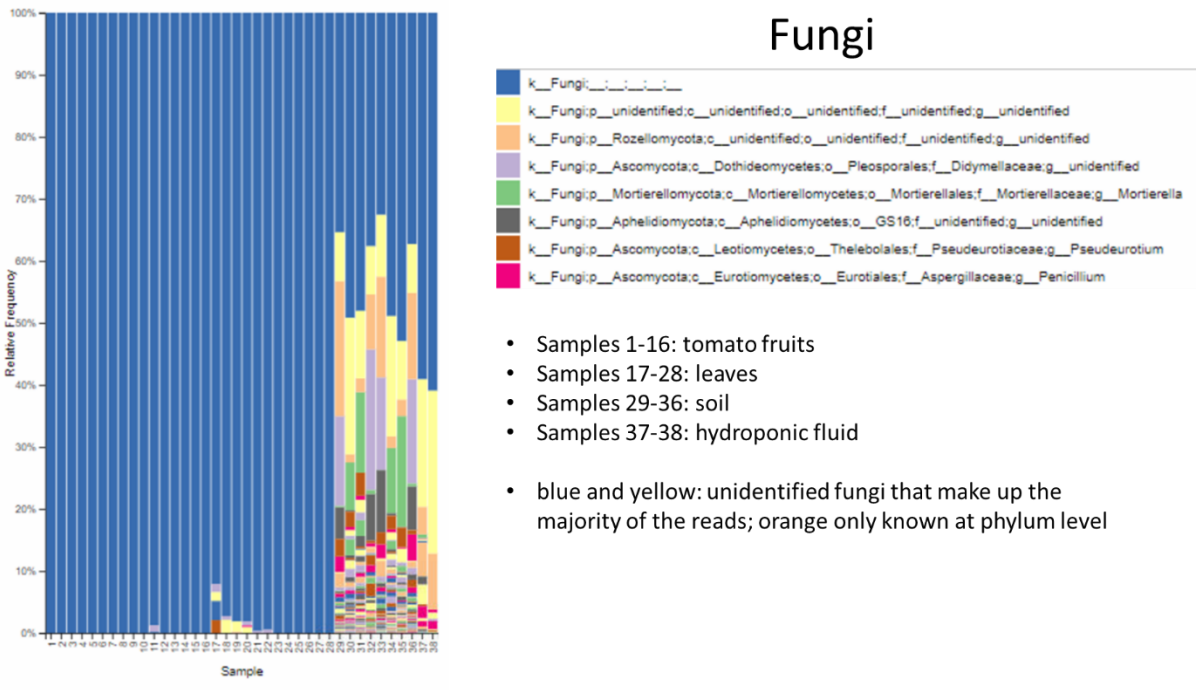


Figure 14 Taxa-barplots of the individual fungi samples. Samples 1 -16 are from tomato fruit; samples 17 to 28 from tomato leaves, samples 29 to 36 soil; samples 37 and 38 hydroponic fluid and coconut substrate.

TIM analysis of tomato fruit samples

TIM analysis was not done, due to the fact that the abundance of bacterial DNA in the samples compared to the tomato DNA was too low, in spite of the use of blockers in repeated runs. No useful information could be expected to be obtained from TIM experiments with such low amount of DNA in the samples.

4. Discussion

Microbiome diversity differences between organic and non-organic samples

The first hypothesis in this pilot study was to test whether organic and non-organic management systems lead to differences in microbiomes of soil/substrate and in or on edible plant parts.

The pilot study shows an overall larger alpha diversity in samples from organically grown tomatoes compared with those from non-organically grown tomatoes. The Shannon index and the Faith's phylogenetic diversity of bacteria on tomato fruits is larger in the organically grown tomatoes. Faith's PD is also larger in bacteria and fungi on organically grown tomato leaves. The observed number of features is also higher in bacteria samples from organically grown tomato fruits and leaves, and also from fungi on leaves. Additionally, an overall significant difference in beta-diversity was found between bacteria samples from organic and non-organic grown tomato fruits and leaves (not for the fungi on leaves) supporting the above result. However, the beta-diversity analysis shows that there is a large diversity between the four companies as well which could possible explain this result. Especially one organic company seems to be very different from one non-organic company (see Figure 8).

Pielou's evenness index is lower in bacteria samples from organically grown tomato fruits and leaves, indicating that in the organically grown samples a smaller number of species are dominant.

These data suggest that the first hypothesis can be partly confirmed. Differences between bacteria and fungi samples from organic and non-organic grown tomato fruits and leaves have been found. However, beta-diversity indicated as Jaccard dissimilarity for bacteria on tomatoes grouped growers according to rootstock used (see Figure 8), independent from management differences. Further research should establish more insight and information about whether these differences in diversity are caused by variations between growers that are specific to organic or non-organic growing methods or other factors (e.g. rootstock) that are different between the growers. For instance Rodriguez et al. report on substantial effects of soil or hydroponic cultivation methods on bacterial diversity, community structure, and microbiota related flavour and aroma compounds in tomato fruits (Escobar Rodríguez *et al.* 2021). Additionally, effects of growth season and harvesting time points have been found on microbiota communities of various fruits (Goforth *et al.* 2024). Future study should include a larger number of organic and non-organic growers into the study, carefully record cultivation methods, and include more samples per grower.

Effect of eating microbiota on human gut microbiome

Diet has a major impact on the composition of the human gut microbiome. Raw fruits and vegetables contain large amounts of microorganisms which might therefore be the main external source of microbiota for the human gut (Soto-Giron *et al.* 2021). People consuming more diversity of fruits and vegetables and more frequently have been found to have a more diverse microbiome (Wicaksono *et al.* 2023). Studies also report rapid shifts in gut microbiome composition due to changes in diet, for instance after 5 days of animal based diet (Sheflin *et al.* 2017). The soil microbiome is an

important reservoir of the plant microbiome and is therefore eventually connected with the gut microbiome as well. Studies have shown that naturally occurring ecosystems support more complex microbiome communities than intensively managed systems (Wassermann *et al.* 2019).

In our pilot study we can only support the evidence for a higher microbiome diversity and a higher number of observed features in organically grown tomato fruits compared with non-organically grown tomato fruits. Because the abundance of bacteria in the samples was too low for the TIM analysis we were not able to further study the ability of these microbiota to survive the stomach and actually reach the intestines. Recent literature however shows the first evidence that microbiota can in fact survive passage through the gastrointestinal tract (Mantegazza *et al.* 2024, Mantegazza *et al.* 2023).

Study limitations

This pilot study has encountered several setbacks and limitations. First of all, the plan was to use the same batch of seeds for the tomatoes in all experiments. This turned out not to be feasible due to certain privacy regulations in both the organic as well as the non-organic growers.

Secondly, the non-organic growers were highly averse to possible contamination risks. Therefore researchers from the Louis Bolk Institute were not allowed to enter the glass houses to manage the experiments and do any measurements themselves. The researchers were dependent on the personnel of the growers to conduct the measurements which could then not be checked.

Thirdly, it was hard to find any growers to participate in the pilot study. Due to the low number of participating growers, and the inability to use the same batch of seeds and rootstock it was impossible to draw clear conclusions, therefore the here presented data should be seen as indicative only.

In hindsight, the choice for tomato as a crop grown both organically and non-organically of which produce is consumed raw, turned out to be an unfortunate one.

Additionally, the processing of the samples by Baseclear turned out to be suboptimal. The methods used by Baseclear should have been able to prevent the measurement of mitochondrial and chloroplast DNA, however this turned out not to be the case. A large percentage of the DNA measured in tomato fruit and leaf samples was from the chloroplast and mitochondria overshadowing other sources of DNA. In contrast to the experimental plan no measurements were performed on the tomato seeds but measurements of tomato leaves were done instead.

Furthermore, it was not possible to perform the TIM analyses because the abundance of bacterial DNA in the samples compared to the tomato DNA was too low.

Future perspectives

Recent literature and the results of the pilot study suggest that much more knowledge about the effects of different growing conditions such as culture media, soil types, soil management methods, pesticide on the microbial composition of fruits and vegetables is needed. The effects of these conditions on microbial compositions can then be compared between organic and non-organic growing systems, allowing a

further teasing out of the size of the various conditions and the contribution of organic and non-organic to it. An obvious starting point seems to be to further elucidate differences between soil and hydroponic grown fruits and vegetables because literature suggests large effects on microbial composition. Another interesting starting point could be to compare the microbiome of locally grown fruits which can be harvested in riper stages with fruits harvested in distant countries which need to be harvested in earlier stages of ripeness. A third area of research could be to compare the microbiome composition of vegetables grown in monoculture systems versus agriculture in diverse systems. First suggestions in this direction are results from Gao and Zhang showing the effect of companion planting on microbiota composition of soils (Gao and Zhang 2023).

5. Conclusion

This pilot study has shown that it is difficult to successfully organize and conduct a study with both organic and non-organic tomato growers in the Netherlands. The data we could collect suggests differences in bacterial and fungal microbiota composition of tomato fruits and leaves between organic and non-organic growers. However, large differences have also been found between the four growers which might be a major confounder. Recent literature shows that much more knowledge is needed on possible relationships between plant and rootstocks, growing conditions, soil microbiota composition, plant microbiota composition and human gut microbiota composition and eventually human health.

Further, it is advised to consider such further study in crops with no or less phytosanitary restrictions, and to start organizing sample collection from the seed and rootstock/plant producers onwards in the production chain, in order to ensure availability of samples from seeds used and same rootstocks/varieties to minimize differences other than the intended contrast in crop management: organic versus conventional.

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7. Appendix 1. Sampling protocol microbiome tomato

Materials supplied by LBI

- Thin bags with labels for fruits and vegetables
- Paper towels for the leaf samples
- Labels
- Thick plastic bag for ground monster/Flask for feeding liquid at substrate.

You get 2 locations within the greenhouse per sample, for example 2 locations marked with a Shadow Leaf. You just take 1*25 grams of leaf material, but maybe it doesn't fit in 1 bag so you get 1 extra bag per sample.

Supply materials yourself

- Sample material. Prefer breeds which are often given rough (cocktail/ cherry /"snack" tomato).
- Scoop/dropper for soil monster
- Possibly a meal for cutting leaf monsters

The required monsters:

- Fruits, 1000 grams
- Leaf material, 25 grams
- Substrate, 500 gram soil at organic level / 500 ml feeding solution at acceptable level

Check location in the glasshouse

1. Divide your greenhouse into sun (northern side of an alley) and shade (usually the southern side of the alley)
2. Select 1 series of 5 plants on the sunny side and 1 series of 5 plants on the shadowy side (10 plants in total) for sampling the same breeds. Important: the plants you have selected are the same combination of rootstock + graft .
    a. Mark 1 series of 5 plants on the sun.
    b. Mark 1 series of 5 plants on the shade
    c. Keep a minimum distance of 2 metres from a main concourse through the department.
    d. Also use these same plants for the leaf sampling.

Fruit samples

1. For each series of 5 plants, collect 1 kg of vegetal fruits, ideally 200 grams per plant. Collect these 5*200 tomatoes in 1 bag with the correct label on it.
2. Allow the tomatoes to cool slightly in the refrigerator (>4 and <7 ˚ C) until they have been collected for transport. Ideally the tomato samples were picked on the same day that the samples were stored in the freezer prior to further processing.
3. Fill out the logbook.

Leaf samples

1. For each series of 5 plants, add 25 grams of leaf material, ideally 5 grams per plant. A leaf the size of your hand weighs approximately 2 grams. So about 3 leaves per plant: 15 leaves in total per sample.
2. Check sample weight. The minimum is 25 g.
3. Put each leaf sample between 3 layers of absorbent paper, for example paper towels. Make sure the leaves are covered.

4. Put the leaf samples in the marked bag.
5. Let the leaf sample bags cool down (>4 and <7°C) until they are collected. Ideally the leaf samples were taken on the same day that these samples were collected by us from your site.
6. Fill in the logbook.

Substrate soil (solid)

- Take soil samples from under the same plants where you also took the leaf and fruit samples from. Make sure you collect at least 500 grams of soil per sample.
- Use a spoon and take a soil sample within 10 cm of the tagged plants.
- Put the samples in the thick plastic bags, do not remove labels already in the bags.
- Store soil samples in the cooler (>4 and <7°C). Ideally the soil samples are taken on the same day that the samples are to be collected by us.
- Fill in the logbook.

Substrate feeding medium (rockwool)

- Tap 500 ml of the liquid medium right before it goes into the disinfection unit.
- Fill out the logbook.

8. Appendix 2. Tomato crops sampled

Grower	**ENZA**
Location - department	demonstration facility Duijvestijn
Name rootstock	Maxifort
Name variety	Test hybrid with new virus resistance (dept. 4 row 411)
Tomato type	Vine tomato - medium
Setting	32 bunches
Date sampling	July 26, 2022
Temperature setting daytime ˚C	20
Temperature setting night-time ˚C	16
Temperature realised ˚C	20/16
Additional artificial lighting?	Not applicable
Cooling available?	yes
Actual temperature at sampling ˚C	21.9 °C
Shade screens open/close at … lux	Not applicable
Setting relative humidity	80%
Actual relative humidity	83%
Crop protection in last 14 days before sampling	none
Crop treatment in last 14 days before sampling (general)	Tying up of main shoot (2x), lowering of main shoot top (2x), pruning of leaves and side shoots (2x), harvesting tomatoes
Remarks	(none)

Grower	**Van der Knaap (non-organic)**
Location - department	Honselersdijk department 10
Name rootstock	Estamino
Name variety	Completon
Tomato type	Normal round
Setting	Year round
Date sampling	July 25, 2022
Temperature setting daytime ˚C	20
Temperature setting night-time ˚C	14
Temperature realised ˚C	20 °C daytime / 14°C night-time
Additional artificial lighting?	no
Cooling available?	yes
Actual temperature at sampling ˚C	23
Shade screens open/close at … lux	Not applicable, used only to limit stray light during night-time
Setting relative humidity	75%
Actual relative humidity	86%
Crop protection in last 14 days before sampling	Organic only: *Macrolophus caliginosus* (=predatory bug) and *Eretmocerus eremicus* (=parasitic wasp)
Crop treatment in last 14 days before sampling (general)	Tying up of main shoot, removal of side shoots, harvesting tomatoes
Remarks	(none)

Grower	**De Lepelaar (organic)**
Location - department	Main location
Name rootstock	Maxifort
Name variety	Annamay F1
Tomato type	Cocktail type
Setting	#9
Date sampling	July 23, 2022
Temperature setting daytime ˚C	Windows closing below 20 °C
Temperature setting night-time ˚C	Windows closing below 20 °C
Temperature realised ˚C	24
Additional artificial lighting?	Not available, glass also not chalked for shading
Cooling available?	no
Actual temperature at sampling ˚C	24
Shade screens open/close at … lux	Not available
Setting relative humidity	No setting
Actual relative humidity	No setting
Crop protection in last 14 days before sampling	Not applicable: organic management
Crop treatment in last 14 days before sampling (general)	Tying up of main shoot, removal of side shoots, harvesting tomatoes
Remarks	(none)

(Due to personal circumstances of the grower, detailed data of the relevant crop and growing conditions could not be retrieved.)

Grower	**BioVerbeek (organic)**
Location - department	Location Tussen de Waeg department 3 (Velden)
Name rootstock	Fortamino
Name variety	Brioso
Tomato type	Cocktail truss type
Setting	(no data)
Date sampling	July 25, 2022
Temperature setting daytime ˚C	(no data)
Temperature setting night-time ˚C	(no data)
Temperature realised ˚C	(no data)
Additional artificial lighting?	(no data)
Cooling available?	(no data)
Actual temperature at sampling ˚C	(no data)
Shade screens open/close at … lux	(no data)
Setting relative humidity	(no data)
Actual relative humidity	(no data)
Crop protection in last 14 days before sampling	Not applicable: organic management
Crop treatment in last 14 days before sampling (general)	(no data)
Remarks	(none)

9. Appendix 3. Overview analyses planned and carried out

Tomato production and fresh produce analysis

- Seed lot: **not done**, as seed lots were not available to us.
- Soil and substrate liquids: done
- Tomato leaves: done
- Tomato pulp: done
- Tomato seeds: **not done**, due to high background signal of tomato DNA

TIM analysis

- Digested tomato pulp: **not done**, due to high background signal of tomato DNA
- Digested tomato seeds: **not done**, due to high background signal of tomato DNA

10. Appendix 4. Research questions addressed

1. Does the microbiome contained in the seed lot used for sowing, relates to the microbiome found in the growth medium, plant roots and harvested edible plant parts? ***- Not done, seed lots not available.***
2. Can we find evidence for the "heritability" of the microbiome through seed generations by comparing the microbiome found in the seed in the harvested fruit to the microbiome present in the seed lot used for sowing? ***- Not done, seed lots not available.***
3. Does crop management (organic vs conventional production) produce different microbiomes present in the same crop? ***– Yes, different management practices produce different microbiomes present on and in tomatoes.***
4. Does the growth medium (organic soil versus Rockwool substrate) produce different microbiomes on the same crop in roots or harvested edible plant parts? ***– Yes, different growth media produce different rhizoshere microbiomes.***
5. Is the microbiome found in tomato fruit pulp and in the seeds identical? ***– Not done due to strong background signal from tomato DNA.***
6. Do different microbiomes in tomato fruit pulp or seeds differ in their survival rate to the human gut microbiome? ***– TIM analysis not done.***
7. Can any similarities be found between the microbiome of edible plants parts and the already known species of the microbiome in the human gut? ***– TIM analysis not done.***
8. Can we relate found effects of food microbiome on the human gut microbiome to known beneficial health effects in literature? ***– TIM analysis not done.***

11. Appendix 5. Contribution of authors

Task	van Agtmaal	van den Berg	DeLong	van Es	Keijzer	van der Kolk	van Malland	Venema	van Wietmarschen
Project planning and experimental set-up				x	x		x		
Sample collection		x	x			x			
Microbiome analyses (by BaseClear)								x	
Raw microbiome data processing								x	
Analyses and indices calaculations									x
Interpretation and conclusions	x								x
Writing of the report					x			x	x
Project coordination					x				