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Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

Exploring the Influence of Future Climate Change on Diversity and Composition of Bacterial Communities in Pear Leaves. The Ecotron Experiment

Mwahija Zubery

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

Prof. dr. Francois RINEAU

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*Running title: *Climate Change Effects on Pear Leaf Bacteria*

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ABSTRACT

Background. Climate change increases pathogen issues in fruit cultivation, reducing quality and causing significant crop damage. While beneficial diverse bacteria enhance plant stress tolerance and nutrient uptake, their specific interaction with pear leaves remains poorly understood. Therefore, we hypothesized that exposure to future climate conditions could (1) increase the diversity and (2) shift the structure of bacterial communities in the top, middle, and lower pear leaves.

Methods. In an eight-month Ecotron experiment, twelve pear trees were set to ambient climate 2013 as a control and future climate 2040 as a treatment. The 16S rRNA gene was analyzed from bacterial communities in pear leaves using Illumina sequencing.

Results. Shannon diversity index using amplicon sequence variants assessed bacterial community diversity. The general linear model and post hoc test findings did not support our first hypothesis that future climate did not significantly increase the diversity of bacterial communities in any leaf position ($p > 0.05$). The Analysis of Similarity results supported our second hypothesis that there was a shift in bacterial community structure under future climate, especially in the middle leaf ($R = 0.534$, $P = 0.001$).

Conclusion. Climate factors are important in shaping the structure of bacterial communities in pear leaves. The response of these communities could be influenced by the leaf location. Our research provides valuable baseline data for agricultural practices and ecological studies.

INTRODUCTION

Belgium is Europe's largest pear (*Pyrus communis*) producer and exerts notable economic influence. It is increasing in area, with 'conference' as the main pear cultivar. Over 10,000 hectares, pears represented more than 50% of fruit production in Flanders in 2022 [1]. However, pear orchards face significant challenges due to climate change.

The origins of the climate crisis trace back to the 1800s when human activities associated with the Industrial Revolution have been the primary driver of climate change. Burning fossil fuels such as coal, oil, and gas has increased greenhouse gas emissions, including CO_2, N_2O, and CH_4 in the atmosphere [2]. Notably, CO_2 emissions have risen sharply. Regarding temperature, Europe has experienced a more rapid increase than the global average. Over the last decade, the mean annual temperature across European land areas has been 2.04 to 2.10°C higher than during the pre-industrial era [3]. Climate models project further temperature rises, ranging from 1.2°C to 1.8°C (under the low emission scenario, model the representative concentration pathway RCP 2.6) to 1.3°C to 1.9°C (under the high emission scenario, model RCP 8.5) by 2021 - 2040 [4]. According to precipitation, the latest Intergovernmental Panel on Climate Change (IPCC) report highlights detectable changes in seasonal mean rainfall

across the global land [5]. In Belgium, high confidence exists that precipitation variability will continue to increase, resulting in more intense rainfall events and longer dry spells [6].

These climate conditions affect pear orchards and other agricultural systems. Changes in precipitation patterns pose a dual challenge, with some regions experiencing increased rainfall while others face water scarcity. Rising temperatures can lead to heat stress in pear trees, affecting their growth, fruit quality, and overall productivity [7]. Altered seasonal patterns impact the timing of flowering, fruit set, and harvest. However, warmer temperatures can favor the proliferation of pests and diseases, threatening pear orchards and disturbing plant microbiome balance. The plant microbiome's balance is crucial for maintaining plant health [8]. It refers to the diverse community of microorganisms such as bacteria, fungi, and viruses that inhabit various plant parts, including the roots, leaves, and stems. Microbial community diversity encompasses species richness (the number of different species) and evenness (how evenly distributed those species are), while composition identifies the abundance of different species within a community.

The bacterial communities in the phyllosphere (leaf) mostly belong to the phylum *Proteobacteria*, which contains both beneficial and pathogenic species [9]. These communities are diverse and abundant, with an average of 10^6 - 10^8 cells per square centimeter [10]. Biodiverse communities are equipped to withstand environmental stresses, making understanding their composition and diversity crucial for predicting their responses to climate change [11].

Environmental stresses affect leaf positions differently due to variations in microclimate conditions across the leaf surfaces [12]. In the open field, the top leaf surface experiences more direct sunlight and wind, higher temperatures, and increased UV radiation, making it prone to greater heat stress [13]. In contrast, the bottom leaf surface is typically cooler and more shaded, maintaining higher humidity levels, which can lead to different microbial colonization patterns. However, the middle leaf surface experiences intermediate conditions, with moderate exposure to sunlight, wind, and temperature. These contrasting conditions mean that the top surface may host more UV-resistant and drought-tolerant microbial species, the middle surface supports a mix of species adapted to moderate conditions, and the bottom surface supports communities that thrive in cooler, moister environments, further influencing the composition and function of their respective microbial communities. Thus, whether at the top or ground level, both beneficial and pathogen leaf-associated bacteria play a crucial role in maintaining the balance of their communities under changing climatic conditions.

Pathogenic microorganisms can infect pear plants and cause many diseases [14]. These microorganisms help regulate bacterial community diversity by controlling the growth of specific species. Their impact on different leaves affects the overall community composition. Several studies on various crops, including apple, tobacco, pumpkin, almond, and many more, found that *Pseudomonas syringae* is the most abundant microorganism in these crops [15]. It grows on the surface of the leaves but can also enter through stomata or wounds to inner leaf tissue and, through multiplication, cause disease in intercellular space. Another bacterial pathogen is *Erwinia amylovora*, which causes Fire blight disease that affects pear tree blossoms, shoots, and branches [16]. At 110 degrees above 18.3 °C, an average daily temperature of 15.6 °C, that pathogen causes the infected parts to wilt, turn brown or black, and look like they have been scorched by fire [15]. The disease can spread rapidly and kill the entire tree, leading to crop damage.

However, beneficial bacteria protect the plant from harmful pathogens, improve nutrient uptake, and enhance plant growth [17]. These bacterial communities shield pear leaves against environmental stressors such as high temperatures, low precipitation, and elevated CO_2 levels. Very little is known about the effect of climate change on these communities.

High temperatures and low precipitation can affect the abundance and diversity of beneficial bacterial taxa by altering leaf surface conditions and adaptive mechanisms. Specific taxa, such as *Enterobacteriaceae*, *Xanthomonadaceae*, and *Bacillaceae* are known for their adaptability to diverse environments, may tolerate high temperatures, and maintain their abundance in the phyllosphere [14]. In periods of low precipitation, *Bacillaceae* synthesize osmoprotectants like trehalose and

proline, enabling plants to withstand water scarcity [18]. However, extreme heat stress may lead to shifts in community composition, favoring thermotolerant genera like *Sphingomonas* and *Pseudomonas*, which possess mechanisms such as the production of heat shock proteins and adaptive metabolic responses that enhance their survival and function under high-temperature conditions [19]. These genera are commonly found in pear leaves and enhance plant growth through a plant hormone, indole-3-acetic acid (IAA) production [20].

On the other hand, the global increase in carbon dioxide may have a particularly important effect, too. Elevated CO_2 can shape the phyllosphere bacterial community, but the specific responses depend on plant species and growth stages. Studies have shown that increased atmospheric CO_2 concentrations can lead to shifts in the abundance and diversity of bacterial taxa inhabiting the phyllosphere and endosphere of leaves [12]. Specifically, bacterial families such as *Xanthomonadaceae* and *Enterobacteriaceae* have been observed in the rice phyllosphere to exhibit significant responses to elevated CO_2 levels, with high relative abundance and changes in community dynamics [12, 21]. Overall, the diversity and composition of phyllosphere bacterial communities are influenced by a complex interplay of environmental factors, shaping the structure and function of microbial communities on plant leaves. Further research will enhance our understanding of these intricate interactions.

This study aims to understand how bacterial community diversity and composition differ between pear leaves exposed to current versus future climate conditions. In particular, we want to understand how future climate conditions may impact the types and abundance of microorganisms in the leaves. The objectives were to (1) investigate the effects of climate conditions on the diversity, composition, and structure of the bacterial community in the top, medium, and bottom leaf phyllosphere and (2) determine the impact of climate change on specific bacterial taxa. We hypothesized that, in the Ecotron, under ambient climate conditions, we expect the bacterial community diversity and structure in the top, medium, and bottom leaves to remain relatively stable. However, future climate conditions could (1) increase the diversity and (2) shift the structure of bacterial communities in the top, middle, and lower pear leaves. Considering the Ecotron, which provides controlled climate conditions, all leaves, whether top, middle, or lower, would experience the same environmental exposure. Additionally, the genera *Pseudomonas* and *Sphingomonas* might dominate the bacterial community. Most species of these genera are beneficial and thermotolerant [22].

To support our hypotheses, we conducted an Ecotron experiment, an enclosed system, and a controlled environment, mimicking a projection of future climate conditions for the 2040 climate, according to the (RCP) 8.5 scenario. The ambient climate of 2013 was used as a control because it had fewer extreme weather events than the 2040 climate. Furthermore, we sampled pear leaves and employed metabarcoding, a powerful technique that identifies microbial communities based on specific DNA regions.

MATERIALS AND METHODS

Study site and sampling

The Ecotron facility is located in Hoge Kempen National Park (50◦ 59′ 02.1″ N, 5◦ 37′ 40.0″ E), Masmechelen, Belgium. This enclosed infrastructure consists of 12 units where environmental conditions are controlled. Each unit hosts a lysimeter with a soil-canopy column, where real-time ecosystem processes can be monitored. On January 17, 2023, twelve pear trees from Proefcentrum Fruitteelt (PCfruit) were planted in six Ecotron units, where air temperatures, atmospheric CO_2 concentration, air relative humidity, precipitation, wind speed, bottom soil temperature, and bottom soil water potential were simulated. Each unit has two pear plants; three simulated the ambient climate of 2013, while the other three simulated the future climate of 2040 (high emissions scenarios, model RCP 8.5). Projections were based on a combination of large and regional climate models, aiming to predict local conditions at a 15 km resolution. The model provided 3-hour-resolution data for air temperature, relative humidity, precipitation, and wind speed. These data were downscaled to half-hour intervals (matching the ecotron's operational time scale) using linear interpolation. Air CO_2 concentrations were adjusted by adding an offset based on real-time CO_2 measurements

from the nearby Integrated Carbon Observation System (ICOS), projected levels for 2040 - 2045 (+133 μmol.mol−1). Soil water potential and air temperature followed field measurements from the nearby ICOS station: an ambient temperature and an elevated +2.0°C. Additionally, the degree of plant exposure to drought stress was assessed using the Ri ratio, with values below 0.6 indicating water stress conditions.

On September 26, 2023, leaf samples (n = 36) were collected from pear trees in the Ecotron. A sample calculation for leaves from the twelve pear trees is as follows: From each pear tree, five leaves from the top, middle, and bottom portions totaling fifteen leaves per tree. Then, samples were brought to the laboratory on ice (4ºC) and preserved at -20°C until further processing. Afterward, samples were homogenized by grinding them in liquid nitrogen and then stored at -20°C.

DNA extraction and sequencing

DNA from 10-mg pear leaf samples was extracted using phenol-chloroform isoamyl alcohol extraction according to RNeasy PowerSoil Kit, automated for Magmax. Briefly, samples were lysed using purple garnet beads, Bead Solution (600 μl), and SR1 (60 μl). Then, phenol:chloroform:isoamyl alcohol (600 μl) was added. The samples were set on a Retsch mixer for 10 minutes at 25Hz, then subjected to centrifuge for 2 min, 13,400 rpm. The upper aqueous phase containing nucleic acids was carefully transferred. NucleoMag B-beads (25 μl) and Binding buffer MWA2 (475 μl) were mixed with samples, followed by shaking. Washing blocks were prepared (850 μl MWA3) and (850 μl MWA4), and the elution plate contained 100 μl DNA-free water. Magmax was run at 4413021 DW Blood program for 20 minutes. DNA was quantified and qualified using a Nanodrop spectrophotometer based on a purity of A260/280 and A260/230 ratios.

Extracted DNA was amplified in the V4 region of 16S rRNA genes with the primer set 515F GTGYCAGCMGCCGCGGTAA and 806R GGACTACNVGGGTWTCTAAT generating amplicons of 290 bp [23, 24]. Using the Q5 High-Fidelity DNA Polymerase system, a reaction volume of 25 μl was prepared. The master mix components included 5x Q5 Reaction Buffer, 10 mM dNTP, 10 μM forward and reverse primer, and 2 U/μl Q5 High-Fidelity DNA polymerase. DNA samples were diluted 20x, 40x and 1 μl was added in the well with 7 μl master mix and 17 μl (0.9 μg/μl) Bovine Serum albumin (BSA). The function of BSA is to remove the contaminations such as phenol compounds in the DNA extract [23]. The PCR program consisted of an initial denaturation at 98°C for 3 minutes, followed by denaturation at 98°C for 10 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 7 minutes. All three steps were repeated for a total of 20 cycles. Quality control of PCR products was obtained via gel electrophoresis at 100 V for 30 minutes using DNA molecular weight markers ranging from 200 - 500 bp.

The PCR products were cleaned to remove contaminants and DNA smaller than 200 bp using MagMax. Briefly, 50 μl of 5x concentrated PCR buffer was added to the 25 μl PCR product along with 60 μl of AMPure XP beads. After thorough mixing, the sample was incubated for 5 minutes to enhance DNA binding. Wash blocks with 200 μl of 80% ETOH were prepared twice, followed by elution with 75 μl of 10 mM Tris HCl pH 8.5. The MagMAX was run at AM1839spin DW program for 10 minutes to carry out binding, wash steps, bead drying, and heated elution. Subsequently, gel electrophoresis was performed to check whether the washing steps worked, and we only have double-stranded DNA and no primer dimers, ssDNA, or other contaminants.

Then, a second PCR for attaching Nextera indices (Nextera XT index Kit v2 Set A (FC-131-2001), Illumina, Belgium) was done using the Q5 High-Fidelity DNA Polymerase system. A master mix containing 5x Q5 Reaction Buffer, 10 mM dNTP, 2 U/μl Q5 High-Fidelity DNA polymerase, and nuclease-free water was prepared. To the cleaned 5 μl PCR product, we added 15 μl of master mix along with 2.5 μl of index 7i and 2.5 μl of index 5i. Then, the mixture was short-spined and amplified with an initial denaturation at 98°C for 3 minutes, followed by denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 7 minutes. All three steps were repeated for a total of 20 cycles. The PCR product was cleaned up, and DNA was qualified using gel electrophoresis. Then, 5 μl sample was pooled, diluted to 1.84 ng/μl, and PCR products were quantified using the Qubit 2.0 fluorometer (Invitrogen) and the Qubit dsDNA HS assay kit (Invitrogen). Then, samples were

sent to Novogene for Illumina partial lane sequencing on the NovoSeq6000 PE250.

Bioinformatics and statistics

The raw sequencing data of the 16S rRNA gene was processed in RStudio version 4.3.3 using the DADA2 package to quality filter, denoise, merge, remove chimera, and assign taxonomies. The sequences were clustered into ASVs (Amplicon sequence variants) using the Greengenes and SILVA databases.

The Shannon index based on ASVs determined the diversity and species richness of the leaf bacterial communities using the vegan R package. Shapiro-Wilk test was done to measure the normality in each group. Bartlett's test was used to measure the homogeneity of variances between groups.

The Analysis of Similarity (ANOSIM) examined the differences in leaf bacterial community structure based on their ASVs abundances using the Bray-Curtis dissimilarity metric, implemented through the vegan package. This package provides functions for ecological community analysis, which evaluates the dissimilarity between groups of samples. Furthermore, a general linear model (GLM) was performed to determine the mixed effect of climate conditions and leaf positions on microbial communities, followed by post-hoc tests to explore pairwise differences between subgroups. P-values less than 0.05 were considered significant.

RESULTS

Bacterial community diversity in response to climate conditions

A total of 8694 sequence reads, and 12,240,410 ASVs were obtained from pear leaves after removing low-quality and chimera sequences. The number of ASVs in the samples ranged from 20 to 18059, with an average of 39.10. As shown in **Fig 1**., the Shannon diversity index based on ASVs was used to evaluate the effect of climate conditions on bacterial richness and diversity. The general linear model and post hoc test findings did not support our first hypothesis that future climate conditions did not significantly increase bacterial richness and diversity in any leaf position. Future climate conditions increased bacterial diversity in the top and lower leaves (P = 1), while it decreased it in the middle leaf (P = 0.852). However, these changes were not statistically significant compared to the ambient climate conditions (**Table S1**).

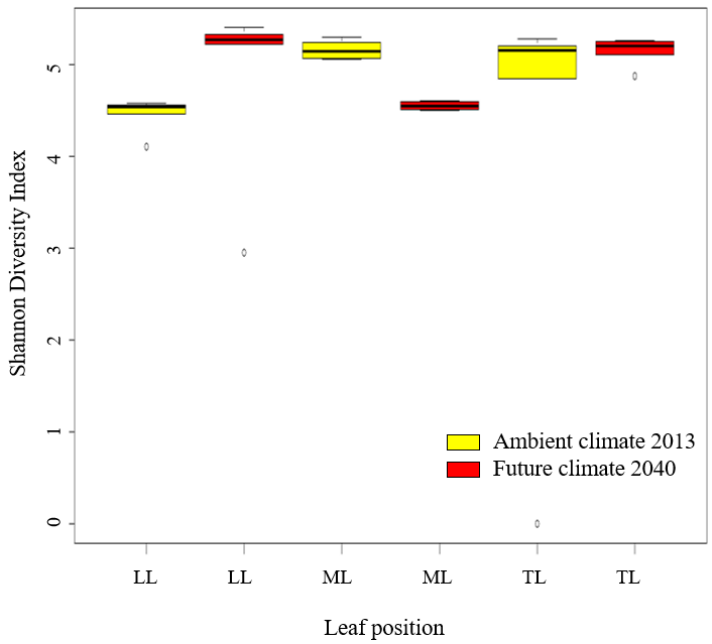

Fig. 1 – Box plot of Shannon diversity indices for pear leaf bacterial community according to climate groups and leaf positions, (n = 36). Ambient climate 2013 (yellow), future climate 2040 (red). LL (lower leaf), ML (middle leaf), TL (top leaf). Post-hoc test, p-value > 0.05

Bacterial community structure in response to climate conditions

The ANOSIM test was assessed using ASV abundances to compare dissimilarities between and within groups. The results indicated that the structure of leaf bacterial communities did not significantly vary across ambient climate 2013 and future climate 2040 (p = 0.967, R = -0.081, **Fig. S1**). However, according to leaf positions, there is a significant difference between climate conditions (R = 0.534, P = 0.001, **Fig. 2**). This finding supports our second hypothesis. The graphical interpretation suggests that future climate conditions have shifted the bacterial community structure, particularly noticeable in the middle leaf. This observation is in comparison with the microbial structure under ambient climate conditions.

R ranges from -1 (if all lowest ranks are between groups) to +1 (if all lowest ranks are within groups). An R-value close to 1 indicates strong separation (significant differences). An R-value close to 0 suggests no significant separation. An R-value close to -1 demonstrates that the groups are more similar than expected by chance. In this study, the positive R-value suggests that the leaf bacterial communities across different climate conditions are more dissimilar than expected by chance.

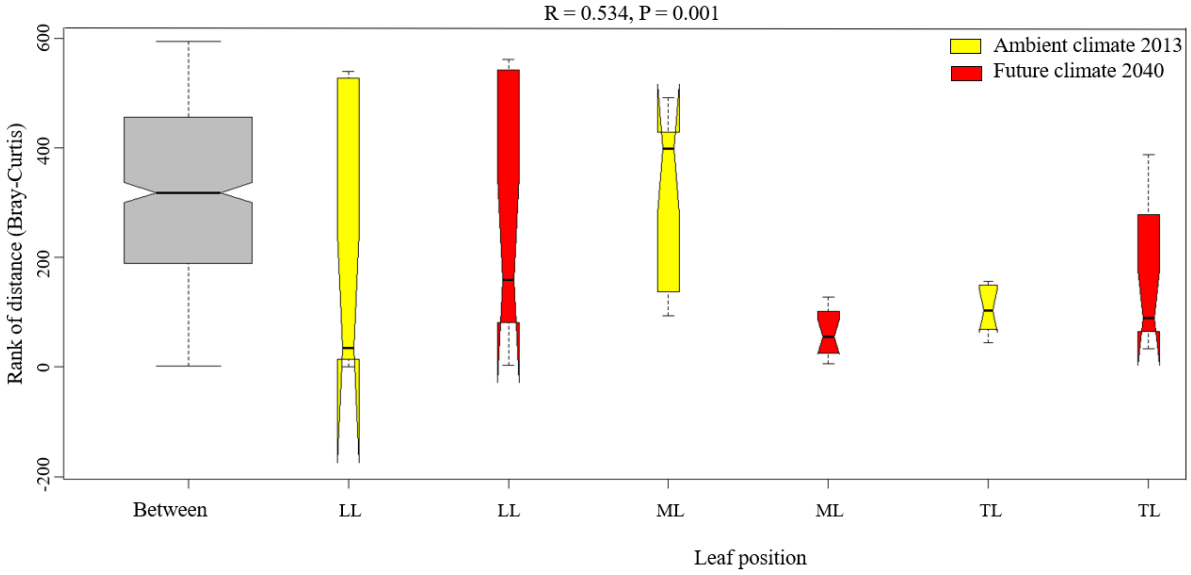

Fig. 2 – Box plot of analysis of similarity (ANOSIM) shows rank of distance for pear leaf bacterial community structure according to climate groups and leaf positions, (n = 36). Ambient climate 2013 (yellow), future climate 2040 (red). LL (lower leaf), ML (middle leaf), TL (top leaf)

Specific bacterial taxa and their response to climate conditions

Relative abundances were used to assess the composition of the bacterial taxa. The *Proteobacteria* was the most abundant phylum, accounting for 50.02% of all bacteria sequences, followed by *Actinobacteria* (8.23 %), *Planctomycetes* (7.25 %) and *Acidobacteria* (6.48 %). Some classes of bacteria had a relative abundance of more than 2%. They were *Gammaproteobacteria* (26.93 %), *Alphaproteobacteria* (20.96 %), *Actinobacteria* (5.62 %), *Bacilli* (3.79 %) and *Deltaproteobacteria* (2.26 %). At the order level, relative abundances were *Sphingomonadales* (11.74 %), *Enterobacteriales* (8.91 %), *Pseudomonadales* (8.71 %), *Betaproteobacteriales* (6.37 %), and *Bacillales* (2.99 %). The most abundant family members were *Sphingomonadaceae* (13.53 %), *Enterobacteriaceae* (10.27 %), and *Pseudomonadaceae* (8.93 %). The most dominant genera were *Pseudomonas* (7.06 %) and *Sphingomonas* (3.69 %). These genera are assumed to be beneficial and commonly found in pear leaves. Based on the general linear model with post hoc tests, future climate conditions significantly increased the *Pseudomonas* relative abundance in the top leaf (P = 0.038, **Fig. 3A**). In contrast, there was no

significant decrease in *Pseudomonas* relative abundance in the middle leaf (P = 0.999). Additionally, the relative abundance of *Pseudomonas* in the lower leaf increased, although not significantly (P = 1). *Sphingomonas* relative abundance did not differ significantly between leaf positions in both climate conditions (p > 0.05, **Fig. 3B**). The information about post hoc tests is found in **Table S2** (*Pseudomonas*) and **Table S3** (*Shingomonas).*

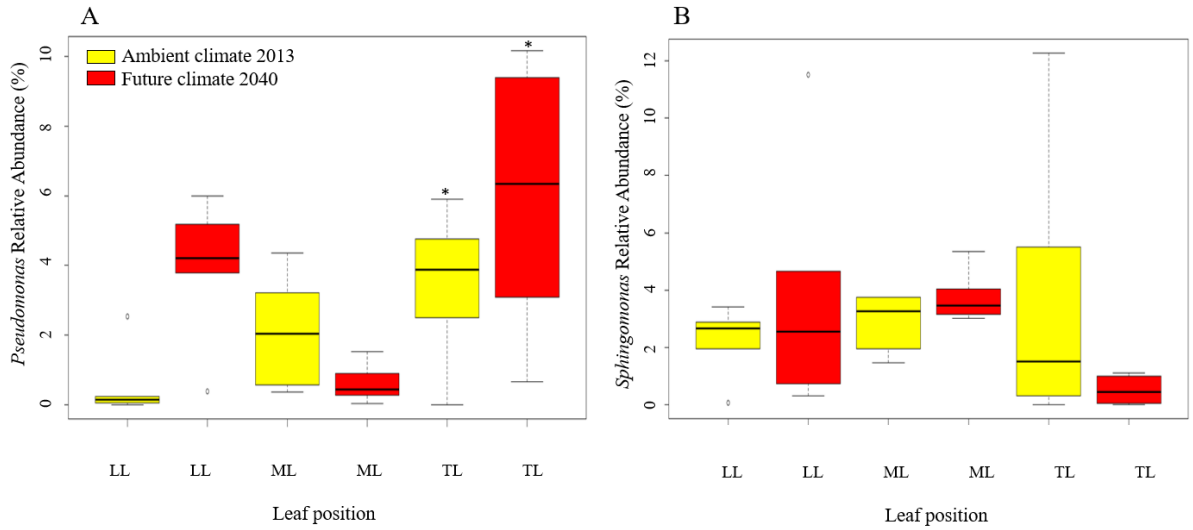

Fig. 3 – Box plots; (A) *Pseudomonas* and (B) *Sphingomonas* genera indicate relative abundance percentages of pear leaf bacterial community according to climate groups and leaf positions, (n = 36). Ambient climate 2013 (yellow), future climate 2040 (red). LL (lower leaf), ML (middle leaf), TL (top leaf). Post-hoc test , * p-value < 0.05

DISCUSSION

This study aimed to understand how bacterial community diversity and composition differ between pear leaves exposed to current versus future climate conditions, specifically focusing on the impact of these conditions on microbial communities across different leaf positions. Our hypotheses anticipated that exposure to future climate conditions could (1) increase the diversity and (2) shift the structure of bacterial communities in the top, middle, and lower pear leaves.

The Shannon diversity index revealed no significant differences in bacterial richness and diversity between ambient and future climate conditions across all leaf positions. Although future climate conditions appeared to increase bacterial diversity in the top and lower leaves and decrease it in the middle leaves, these changes were not statistically significant (**Fig. 1**). This suggests that, within the experimental timeframe and conditions, the diversity of leaf-associated bacteria remained relatively stable despite changes in climate conditions. Such stability in bacterial diversity could imply that these microbial communities are resistant to shifts in climate variables, at least in the short term. The middle leaf had low diversity under future climate because, in the enclosed Ecotron system, this leaf might have experienced more wind and high temperatures. Our findings are inconsistent with the previous study using the FACE (Free Air CO_2 Enrichment) approach; they found an increase in the diversity of bacterial communities from the top rice leaf was significantly affected by elevated CO_2 plus temperature [12]. The FACE approach involves exposing plants to elevated CO_2 levels in an open-air environment, allowing for more natural variations in environmental conditions and interactions with the surrounding ecosystem.

The ANOSIM test demonstrated that the bacterial community structure did not significantly differ between the ambient and future climate conditions when considered as a whole (**Fig. S1**). However, significant differences were observed when considering leaf positions under different climate

conditions. Future climate conditions shifted the bacterial community structure, particularly noticeable in the middle leaf position (**Fig. 2**). Our finding is similar to a previous study, which showed that the high temperature and CO_2 concentrations shifted the composition and structure of the phyllosphere bacterial community [12]. Despite climate change, nutrient content and carbon substrate may impact community structure [26]. Leaves with higher nutrient content (e.g., nitrogen, phosphorus) may support different bacterial taxa than nutrient-poor leaves.

The composition analysis revealed that *Proteobacteria* was the most abundant phylum, followed by *Actinobacteria*, *Planctomycetes*, and *Acidobacteria*. Within *Proteobacteria*, *Gammaproteobacteria* and *Alphaproteobacteria* were the dominant classes. The genera *Pseudomonas* and *Sphingomonas* were particularly noteworthy. These genera are commonly distributed in pear leaves, are thermotolerant, and play a beneficial role in plant health [13]. *Pseudomonas* significantly increased relative abundance in the top leaf under future climate conditions (**Fig. 3**A). This increase suggests that future climate scenarios might favor the proliferation of beneficial bacteria in certain leaf positions, potentially aiding the plant in coping with increased heat stress. In contrast, *Sphingomonas* did not show significant differences in relative abundance between the different leaf positions and climate conditions (**Fig. 3B**). The stability in *Sphingomonas* abundance might indicate its robust adaptability to varying climate conditions, ensuring continued support for the pear tree's health. Our study, consistent with previous research, revealed a high relative abundance of *Sphingomonas ssp.* in response to elevated temperature and CO_2 [12]. The opposite results from a longer-term field study showed the impact of moderate (+2°C) surface warming on the phyllosphere microbiota of plants. Specifically, the leaf samples of *Galium album* from warmed plots determined beneficial bacteria like *Sphingomonas spp.* and *Rhizobium spp*. were less abundant [25]. This suggests that future climatic scenarios could induce more profound changes in microbial community composition due to long-term exposure to environmental factors such as light, temperature, and humidity.

The various effects on bacterial diversity and composition under simulated climate conditions may have several implications. Firstly, this study utilized a relatively small sample size with limited replication within the Ecotron units, which might affect the statistical power and generalizability of the results. Secondly, the project was conducted over a limited timeframe (eight months), potentially not capturing long-term effects and seasonal variations in bacterial communities. Thirdly, the research primarily focused on the diversity and composition of bacterial communities but did not delve deeply into the functional roles of these microbes. Fourthly, it is essential to consider that the enclosed system and controlled conditions of the Ecotron experiment might not fully replicate the complexities of natural orchard environments.

CONCLUSION

These findings reveal that while overall bacterial diversity and composition may not significantly shift, specific leaf positions exhibit marked changes under future climate conditions. The increased abundance of specific genera, such as *Pseudomonas*, in the top leaves suggests an adaptive microbial response to climate change.

In conclusion, While our study indicated varied effects in pear leaf bacterial communities under simulated climate conditions, it provides valuable information for pear agricultural practices and environmental research. Future work should consider increasing the sample size and replication within controlled environments such as the Ecotron to enhance statistical power and result in generalizability. Incorporate metagenomic and metatranscriptomic approaches to analyze the function and activity of microbial communities. Additionally, extending the experimental duration to cover multiple growing seasons would enable capturing long-term effects and seasonal variations in bacterial communities. Furthermore, complementing controlled environment studies with field experiments in actual orchards is crucial to validate findings and ensure their applicability to real-world scenarios. By integrating these recommendations, future studies can provide more comprehensive insights into the dynamics of phyllosphere microbial communities in response to climate change and agricultural practices.

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Author contributions – Prof. dr. Francois Rineau conceived and designed the research. Mwahija Zubery and Nier Su collected samples from the Ecotron. Mwahija Zubery and Vera Claessens performed experiments and data analysis. Mwahija Zubery wrote the paper. Prof. dr. Francois Rineau and Vera Claessens carefully edited the manuscript.

APPENDICES

Table S1 – Pairwise comparison of Shannon diversity indices of bacteria communities, grouped by climate conditions and leaf positions. Comparisons were performed using a General linear model with post-hoc tests. (n = 36)

Contrast	Estimate	SE	df	t.ratio	p.value
Ambient climate 2013 Lower leaf - Future climate 2040 Lower leaf	-0.092	0.548	30	-0.168	1.000
Ambient climate 2013 Lower leaf - Ambient climate 2013 Middle leaf	-0.446	0.548	30	-0.814	0.963
Ambient climate 2013 Lower leaf - Future climate 2040 Middle leaf	0.190	0.548	30	0.347	0.999
Ambient climate 2013 Lower leaf - Ambient climate 2013 Top leaf	-0.699	0.548	30	-1.277	0.795
Ambient climate 2013 Lower leaf - Future climate 2040 Top leaf	-0.688	0.548	30	-1.256	0.806
Future climate 2040 Lower leaf - Ambient climate 2013 Middle leaf	-0.354	0.548	30	-0.646	0.986
Future climate 2040 Lower leaf - Future climate 2040 Middle leaf	0.282	0.548	30	0.514	0.995
Future climate 2040 Lower leaf - Ambient climate 2013 Top leaf	-0.608	0.548	30	-1.110	0.874
Future climate 2040 Lower leaf - Future climate 2040 Top leaf	-0.596	0.548	30	-1.088	0.882
Ambient climate 2013 Middle leaf - Future climate 2040 Middle leaf	0.636	0.548	30	1.161	0.852
Ambient climate 2013 Middle leaf - Ambient climate 2013 Top leaf	-0.254	0.548	30	-0.463	0.997
Ambient climate 2013 Middle leaf - Future climate 2040 Top leaf	-0.242	0.548	30	-0.442	0.998
Future climate 2040 Middle leaf - Ambient climate 2013 Top leaf	-0.889	0.548	30	-1.624	0.590
Future climate 2040 Middle leaf - Future climate 2040 Top leaf	-0.878	0.548	30	-1.602	0.603
Ambient climate 2013 Top leaf - Future climate 2040 Top leaf	0.012	0.548	30	0.021	1.000

Table S2 – Pairwise comparison of relative abundances of genus *Pseudomonas*, grouped by climate conditions and leaf positions. Comparisons were performed using a General linear model with post-hoc tests. (n = 36)

Contrast	Estimate	SE	df	t.ratio	p.value
Ambient climate 2013 Lower leaf - Future climate 2040 Lower leaf	-0.222	3.212	30	-0.069	1.000
Ambient climate 2013 Lower leaf - Ambient climate 2013 Middle leaf	-8.948	3.212	30	-2.786	0.088
Ambient climate 2013 Lower leaf - Future climate 2040 Middle leaf	-7.700	3.212	30	-2.397	0.189
Ambient climate 2013 Lower leaf - Ambient climate 2013 Top leaf	-4.087	3.212	30	-1.273	0.797
Ambient climate 2013 Lower leaf - Future climate 2040 Top leaf	-14.239	3.212	30	-4.433	0.001
Future climate 2040 Lower leaf - Ambient climate 2013 Middle leaf	-8.727	3.212	30	-2.717	0.101
Future climate 2040 Lower leaf - Future climate 2040 Middle leaf	-7.478	3.212	30	-2.328	0.214
Future climate 2040 Lower leaf - Ambient climate 2013 Top leaf	-3.866	3.212	30	-1.204	0.832
Future climate 2040 Lower leaf - Future climate 2040 Top leaf	-14.017	3.212	30	-4.364	0.002
Ambient climate 2013 Middle leaf - Future climate 2040 Middle leaf	1.249	3.212	30	0.389	0.999
Ambient climate 2013 Middle leaf - Ambient climate 2013 Top leaf	4.861	3.212	30	1.514	0.659
Ambient climate 2013 Middle leaf - Future climate 2040 Top leaf	-5.290	3.212	30	-1.647	0.575
Future climate 2040 Middle leaf - Ambient climate 2013 Top leaf	3.612	3.212	30	1.125	0.867
Future climate 2040 Middle leaf - Future climate 2040 Top leaf	-6.539	3.212	30	-2.036	0.347
Ambient climate 2013 Top leaf - Future climate 2040 Top leaf	-10.151	3.212	30	-3.161	0.038

Table S3 – Pairwise comparison of relative abundances of genus *Sphingomonas*, grouped by climate conditions and leaf positions. Comparisons were performed using a General linear model with post-hoc tests. (n = 36)

Contrast	Estimate	SE	df	t.ratio	p.value
Ambient climate 2013 Lower leaf - Future climate 2040 Lower leaf	-1.996	2.117	30	-0.942	0.932
Ambient climate 2013 Lower leaf - Ambient climate 2013 Middle leaf	-1.957	2.117	30	-0.924	0.937
Ambient climate 2013 Lower leaf - Future climate 2040 Middle leaf	-1.688	2.117	30	-0.797	0.966
Ambient climate 2013 Lower leaf - Ambient climate 2013 Top leaf	-0.859	2.117	30	-0.406	0.998
Ambient climate 2013 Lower leaf - Future climate 2040 Top leaf	2.405	2.117	30	1.136	0.862
Future climate 2040 Lower leaf - Ambient climate 2013 Middle leaf	0.038	2.117	30	0.018	1.000
Future climate 2040 Lower leaf - Future climate 2040 Middle leaf	0.307	2.117	30	0.145	1.000
Future climate 2040 Lower leaf - Ambient climate 2013 Top leaf	1.137	2.117	30	0.537	0.994
Future climate 2040 Lower leaf - Future climate 2040 Top leaf	4.401	2.117	30	2.078	0.325
Ambient climate 2013 Middle leaf - Future climate 2040 Middle leaf	0.269	2.117	30	0.127	1.000
Ambient climate 2013 Middle leaf - Ambient climate 2013 Top leaf	1.099	2.117	30	0.519	0.995
Ambient climate 2013 Middle leaf - Future climate 2040 Top leaf	4.363	2.117	30	2.060	0.334
Future climate 2040 Middle leaf - Ambient climate 2013 Top leaf	0.830	2.117	30	0.392	0.999
Future climate 2040 Middle leaf - Future climate 2040 Top leaf	4.094	2.117	30	1.933	0.403
Ambient climate 2013 Top leaf - Future climate 2040 Top leaf	3.264	2.117	30	1.541	0.641

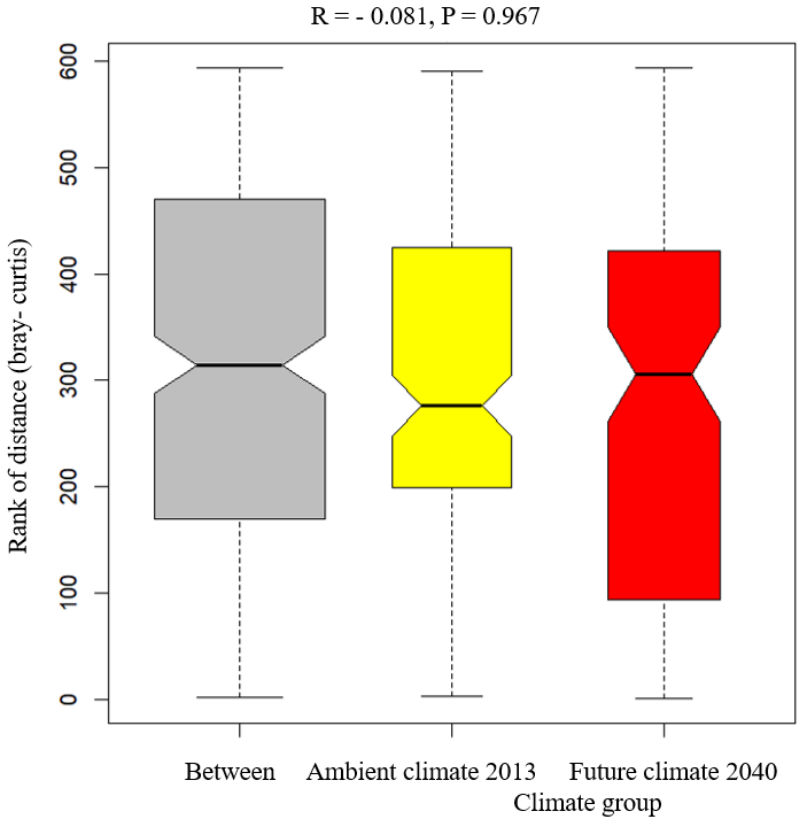

Fig. S1 – Box plot of analysis of similarity (ANOSIM) shows the rank of distance for pear leaf bacterial community structure according to climate groups and leaf positions, (n = 36). Ambient climate 2013 (yellow), future climate 2040 (red)