The bacterial community of the European spruce bark beetle in space and time

Short title: The bacterial community of *Ips typographus*

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Abstract

The European spruce bark beetle *Ips typographus* (L.) is a pest causing severe damage to Norway spruce-dominated forests in Europe. Microorganisms play an essential role in the species life history, including nutrition, fitness as well as in overcoming host defenses. Here, we performed high-throughput 16S rRNA metabarcoding of *I. typographus* across different populations in Europe. We investigated four postglacial refugial areas in Europe and focused specifically on a current bark beetle hot spot in the Dolomites where we compared populations with different epidemiological phases (outbreaking vs. non-outbreaking) and across different seasons (pre-overwintering vs. overwintering). Our results show that the bacterial community structure varied among populations from the refugial areas and geographic regions within the Dolomites. We found a significant difference in the bacterial community between pre-overwintering and overwintering individuals, highlighting a potential role of the microbiome in *I. typographus* overwintering but we did not find differences between epidemic and endemic populations. The genera *Erwinia* and *Pseudoxanthomonas* - previously reported for their role in nutrition and protection from conifer defense compounds - were present in every individual across all populations, suggesting that these taxa form the bacterial core community of *I. typographus*. Furthermore, several additional bacterial taxa occurred in all populations, but with variable frequencies within and between individuals. This study highlights a complex interaction of various bacterial taxa across different regions and ecological phases of *I. typographus* populations and provides new insights into the role of microorganisms in the biology of this important pest species.

Key words: Bark beetle, Curculionidae, *Ips typographus*, forest pest, bacterial diversity, microbiome, endosymbionts, *Erwinia, Pseudoxanthomonas, Spiroplasma*

Introduction

The European spruce bark beetle *Ips typographus* (L.) (Coleoptera: Curculionidae, Scolytinae) is one of the most destructive forest pests in Europe, causing important ecological and economic disturbances over the last decades (Schelhaas et al. 2003; Wermelinger 2004; Hlásny et al. 2017). In Europe, *I. typographus* has a wide geographic range overlapping with that of its main host, Norway spruce *Picea abies* (Karsten) (Pfeffer 1995). Phylogeographic studies showed that *I. typographus* survived Pleistocene glaciation events in different glacial refugia which influenced its population structure (Stauffer et al. 1999; Bertheau et al. 2013). In endemic phases, *I. typographus* breeds and develops within the phloem of weakened and dying trees, especially wind-thrown and drought-stressed trees with impaired defenses, whereas in epidemic phases when population outbreaks occur, this beetle can colonize and kill also apparently healthy trees (Schroeder 2010; Netherer et al. 2015; 2021). In 2018, the storm Vaia destroyed more than 410 km^2 of forests, mainly Norway spruce, in the Southern Alps of north-eastern Italy and southern Austria (Chirici et al. 2019; Udali et al. 2021). Moreover, heavy snow falls affected the Dolomites region in the years 2019 and 2020. These forest disturbance events provided ideal conditions for *I. typographus* reproduction and development and resulted in a dramatic bark beetle outbreak in the Southern Alps (Nardi et al. 2022).

Insects are associated with various microorganisms that provide physiological and ecological benefits to their hosts (Douglas 2015; McCutcheon et al. 2019; Jang & Kikuchi 2020). They play a significant role in various aspects of insect life histories, including nutrition, development, morphogenesis, behavior, and immunity (Engel & Moran 2013; Hosokawa & Fukatsu 2020). In particular, microorganisms are of high significance for phytophagous insects. For instance, despite having endogenous detoxification systems, insects often use associated microbes to detoxify protective plant secondary metabolites (van den Bosch & Welte 2017; Itoh et al. 2018).

Bacterial symbionts also play a significant role in the life histories of numerous bark beetles, for example in the economically important genera *Ips* and *Dendroctonus*, by contributing to the protection of beneficial fungal symbionts, nutrition, detoxification, production of pheromone-like molecules, and protection against pathogens through the production of antimicrobial compounds (Six 2013; García-Fraile 2017). For instance, *Erwinia typographi* isolated from *I. typographus* showed resistance to high concentrations of the monoterpene myrcene, highlighting a potentially important role of this bacterium in defense against terpenoids and phenolic compounds (Skrodenytė-Arbačiauskienė et al. 2012). Similarly, various bacterial associates belonging to the genera *Serratia*, *Pseudomonas* and *Rahnella* were found to reduce concentrations of monoterpenes under controlled conditions in mountain pine beetles of the genus *Dendroctonus* (Boone et al. 2013). Moreover, *Actinobacteria, Bacillus, Brevundimonas, Methylobacterium, Paenibacillus, Pseudomonas, Pseudoxanthomonas, Serratia, Sphingomonas* and *Stenotrophomonas* were described to support degradation of cellulose in different bark beetle species (Morales-Jiménez et al. 2012; Hu et al. 2014). The genus *Pseudomonas* has been isolated from *I. typographus* individuals at different life stages and was shown to play a potential role in nutrient provisioning, protection against pathogens, and degradation of toxic compounds (Peral-Aranega et al. 2020). Therefore, bacteria might assist the successful colonization of trees and might therefore play an important role in mass outbreaking events of *I. typographus.*

Although our understanding of the bacterial community structure of *I. typographus* has increased in recent years (Chakraborty et al. 2020; Fang et al. 2020; Veselská et al. 2022; Yu et al. 2022), surprisingly little knowledge exists about the taxonomic composition and diversity across different geographic regions, epidemiological and overwintering phases. Here, we first characterize the taxonomic composition and core-microbiome of *I. typographus* by characterizing the bacterial communities of different populations from a large area of Europe,

including the four major refugial areas, i.e. Apennines, the Dinaric Alps on the Balkan Peninsula, the Carpathian Mountains and in the Russian plain (Schmidt-Vogt 1977; Tollefsrud et al. 2009). Subsequently, we focus particularly on the Dolomites (Northeastern Italy and Southern Austria), where currently an outbreak of this beetle occurs. In this region, we (1) compare the microbial composition of endemic and epidemic (non-outbreaking vs. outbreaking) populations to investigate the role of bacteria in the population dynamics of *I. typographus* and (2) investigate the potential role of bacteria in overwintering by comparing the bacterial communities of populations in different overwintering phases.

Materials and methods

Sample collection

Adults of *Ips typographus* were collected from seven geographic regions. Populations from the Apennines (Abetone – Italy, Abe), the Dinaric Alps of Croatia (Vrhovine, CR), the Carpathian Mountains of Romania (Belis, RO), and the East European plain (Alexandrov – Russia, RU) were included to assess differences in the microbial community across a broad geographic scale and evaluate potential effects of historic events during the Pleistocene (Figure 1; Table S1). Moreover, we focused specifically on populations within the Dolomites that were affected by the storm Vaia in 2018, namely four populations from Eastern Tyrol (Austria; ET1-ET4), three populations from South Tyrol (Italy; ST1-ST3) and six populations from Veneto (Italy; VT1-VT6) (Figure 1). To compare the bacterial communities between epidemic and endemic populations, the four populations in Eastern Tyrol were collected in different epidemiological phases in summer 2020 (two endemic, ET2 and ET4, and two epidemic sites, ET1 and ET3). The populations from Veneto were sampled before and during winter of 2019 and 2020, to compare the microbial community from pre-overwintering and overwintering

populations. From each population, single adult beetles were collected from different galleries to avoid the analysis of siblings. Live beetles were transferred to absolute ethanol and stored at -20 °C. Detailed information about the localities are listed in Table S1.

DNA extraction and sequencing

DNA was extracted from single *I. typographus* individuals using the Sigma-Aldrich GenElute Mammalian Genomic DNA Miniprep Kit (Saint Louis, Missouri, USA) following the manufacturer's protocol. DNA was quantified with the Invitrogen™ Qubit® 1X dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, USA) and quality was checked with a Nanodrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA). The bacterial communities of 192 individuals were amplified using barcoded primers 515f and 806r which amplify the V4 region of the 16S rRNA gene (Klindworth et al. 2013) and sequenced on an Illumina MiSeq platform using 2×250 bp paired-end chemistry by a commercial provider (StarSEQ GmbH, Mainz, Germany). Moreover, four no template controls were included as negative control. Raw reads will be deposited in a public database upon acceptance of the manuscript.

Data analysis

The raw reads were analyzed using the QIIME2 pipeline (version 2021.4). Due to the poor quality of the reverse reads, only the forward reads (which contain almost the entire V4 amplicon) were used for the subsequent analyses. DADA2 was used for quality filtering, denoising, and Amplicon Sequence Variant calling, using the plug-in q2-dada2 with the denoise-single method. Sequences were then clustered into operational taxonomic units (OTUs) at 97% identity, using the plug-in qiime vsearch cluster-features-de-novo. Taxonomic assignment was done against the SILVA database version 138 (Quast et al. 2012). OTUs identified as chloroplasts, mitochondria, archaea, and eukaryotes were filtered out along with

rare OTUs (i.e., singletons and OTUs represented by < 10 reads). The resulting OTU table was then used for the subsequent analyses.

Data analysis was done using R Studio (version 4.1.2). Taxonomic composition was determined using the 'phyloseq' package (McMurdie & Holmes 2013). Difference in abundance were compared using the 'MaAsLin2' package (Mallick et al. 2021) running a longitudinal feature-wise analysis based on a multivariable regression model to test for the differentially abundant OTUs between pre-overwintering and overwintering populations of Veneto. Furthermore, shared and unique OTUs of the different geographic locations were identified, considering only OTUs with 10 or more reads per sample. Visualization of the intersecting and unique OTUs was carried out using the 'UpSetR' package (Conway et al. 2017).

Bacterial diversity was analyzed using the packages 'phyloseq' (McMurdie & Holmes 2013) and 'vegan' (Oksanen et al. 2015), after rarefying samples to an even sampling depth of 4,500 reads. The alpha diversity indices Chao 1 and Shannon's diversity index as well as Good's coverage (to validate the representativeness of the sequencing effort) were determined for each sample. The normality of distribution was evaluated using the Shapiro-Wilk test followed by ANOVA and Tukey's Honest Significant Difference (HSD) post-hoc test for differences in bacterial diversity among populations and localities. Additionally, Welch's t-test was used to test for differences in diversity between endemic and epidemic populations as well as between pre-overwintering and overwintering populations.

Beta diversity was analyzed using Canonical Analysis of Principal coordinates based on Bray-Curtis dissimilarity using the 'CAPSCALE' package (Oksanen et al. 2015) to determine differences in community structure among the different geographical locations. Permutation multivariate ANOVA (Adonis) was used to assess community structure

differences among different geographical locations at 5,000 permutations followed by a pairwise comparison. Furthermore, hierarchical clustering of samples collected from the Dolomite region was performed based on Bray-Curtis dissimilarity using the 'ggtree' package (Yu et al. 2017).

Results

Predominant taxa in the bacterial community of Ips typographus

After quality control and sequence filtering, a total of 7,342,526 reads ranging from 4,505 to 69,545 reads per individual were retained (Table S2). These were clustered into 8 – 373 OTUs per individual. These OTUs comprised 37 bacterial phyla, 78 classes, 184 orders, 310 families, and 590 genera. The Good's coverage was greater than 99.5% in all 192 samples and all rarefaction curves reached a plateau, indicating that the sequencing coverage was sufficient to capture the bacterial communities of all samples (Figure S1).

The bacterial community composition of *I. typographus* revealed ten major bacterial phyla. The most abundant phyla were Pseudomonadota (formerly Proteobacteria, 25.1%), Bacteroidota (formerly Bacteroidetes, 17.93%), Bacillota (formerly Firmicutes, 11.2%), and Actinobacteria (6.18%), while Myxococcota, Bdellovibrionota, Planctomycetota and Abditibacteriota were present at lower abundance across all localities (Figure 2A). At the genus level, 15 bacterial taxa were present in most of the studied localities, including *Acinetobacter, Allorhizobium-Neorhizobium, Brevundimonas, Chryseobacterium, Erwinia, Izhakiella, Mycobacterium, Novosphingobium, Pseudomonas, Pseudoxanthomonas, Sphingobacterium, Spiroplasma, Williamsia* and *Wolbachia* (Figure 2B). *Erwinia* and *Pseudoxanthomonas* were the only genera present in every individual from all populations and can therefore be considered the core bacteria of *I. typographus*.

Differences in bacterial communities across geographic regions

Overall, 17 OTUs were shared among all populations, which belonged to the genera *Erwinia, Pseudoxanthomonas, Acinetobacter, Spiroplasma, Pseudomonas, Chryseobacterium, Sphingobacterium,* and *Allorhizobium-Neorhizobium*. Additionally, ten OTUs were common in all populations except Russia (Figure 3) which included the genera *Wolbachia, Tyzzerella, Williamsia,* and *Endobacter*. In contrast, the majority of OTUs were exclusively present only in single regions: 134 in the four populations in Eastern Tyrol, 191 in the three populations from South Tyrol, 274 in the six populations from Veneto, 100 in Abetone, 41 in Romania and 120 in Croatia and 135 in Russia (Figure 3). At the genus level, seven genera were shared among all localities and populations, which included *Acinetobacter, Chryseobacterium, Erwinia, Pseudoxanthomonas, Pseudomonas, Sphingobacterium* and *Spiroplasma* (Figure S2). Most genera were exclusively present only in single populations: 18 in Eastern Tyrol, 26 in South Tyrol, 35 in Veneto, seven in Abetone, seven in Romania and five in Croatia and 25 in Russia (Figure S2).

The average bacterial species richness based on the Chao 1 index ranged from 88.9 in Eastern Tyrol to 180 in Croatia (Figure 4). Overall regions showed a statistically different bacterial species richness (ANOVA, $F= 10.69$, $df= 6$, $p < 0.001$). Pairwise comparison of species richness showed significant difference between several localities (Table S3). Moreover, bacterial diversity based on the Shannon index also differed significantly between localities (ANOVA, $F= 17.02$, $df= 6$, $p < 0.001$; Supplementary Tables S4). Beetles from Veneto had the highest Shannon index of 3.39 whereas individuals from Eastern Tyrol had the lowest of 1.74 (Figure 4). The bacterial community composition of *I. typographus* differed significantly between and within localities. This was confirmed by a distinct segregation and a significant difference among the geographical regions (PERMANOVA, $df= 6$, $F=8.46$, $R^2=0.21$, $p <$

0.001; Figure 5A; Table S5). The bacterial species richness showed a significant difference among the different refugial areas (Chao1: ANOVA, F= 15.07, df= 3, $p < 0.0003$), whereas the species evenness was not significantly different among refugial areas (Shannon: ANOVA, F= 1.06, df= 3, $p < 0.377$). Moreover, the hierarchical clustering based on Bray-Curtis revealed a complete segregation indicating that each region has its unique bacterial community structure (Figure 5B).

Fine-scale sampling within the Dolomites allowed the comparison of the bacterial communities of different *I. typographus* populations on a smaller scale. Overall, the bacterial species richness was significantly different between populations from the Dolomites (Chao1: ANOVA, F= 15.53, df= 2, $p < 0.001$). Pairwise comparisons showed a significant difference between ET and ST ($p < 0.03$), ST and VT ($p < 0.04$), and populations from ET and VT had a significantly different bacterial diversity ($p < 0.0001$; Figure 3). Bacterial species evenness was significantly different among the Dolomites regions (Shannon: ANOVA, F= 63.15, df= 2, $p < 0.0001$). Pairwise comparisons showed no significant difference between ET and ST ($p < 0.369$) but a significant difference between ET and VT ($p < 0.0001$) and VT and ST ($p < 0.0001$). These results were confirmed by a PERMANOVA based on Bray-Curtis dissimilarity, which revealed a significant difference among the bacterial community structure of the Dolomites region. The hierarchical clustering showed the segregation among the different Dolomites regions which was strongly pronounced in the case of VT populations when compared to ET and ST populations (Figure 5).

No difference between the microbial communities of epidemic and endemic populations

To investigate potential differences in the bacterial community composition between populations in different epidemiological phases we compared the microbial communities

between endemic (ET2 and ET4) and epidemic populations (ET1 and ET3) from East Tyrol on a small local scale. The bacterial community composition was similar between the epidemic and endemic populations at both phylum and genus levels (Figure 6A; 6B). The bacterial species richness and diversity were similar between groups (Welch's t-test: Chao1, t = 0.49196, df= 37 p = 0.774; Shannon, t = 0.28289, df = 412, p = 0.955). Moreover, Canonical Analysis of Principal coordinates based on Bray-Curtis dissimilarity showed a strong overlap between the endemic and epidemic populations (Figure 6C). This was confirmed by Adonis multivariate analysis of variance, showing that there was no significant difference between the bacterial communities of endemic and epidemic populations of *I. typographus* (PERMANOVA, df= 1, F=0.86, R2=0.018, p = 0.529). These results indicate that the bacterial community is rather influenced by the geographical origin than the epidemic status. However, several taxa appeared in different abundance between epidemic and endemic populations. Most strikingly *Pseudoxanthomonas* and *Spiroplasma* were more abundant in epidemic populations, whereas *Erwinia* was more abundant in endemic populations (Figure 6B).

Different bacterial communities between pre-overwintering and overwintering populations

To study the influence of overwintering on the bacterial community composition, we compared the microbial communities between the pre-overwintering populations VT2, VT3 and VT6 (which were sampled in August and September 2020) and the overwintering populations VT4 and VT5 (which were sampled in December 2019 and January 2020). Although the bacterial composition on the phylum level was similar between pre-overwintering and overwintering populations, there were differences in the abundance where *Bacillota* was more abundant in pre-overwintering and *Bacteroidota* in overwintering populations (Figure 7A). Especially, the relative abundance of the genera *Spiroplasma*, *Tyzzerella* and *Lactococcus* was significantly higher in pre-overwintering populations (Figure 7B, Table S6). In contrast,

the genera *Lactobacillus*, *Bacillus*, *Listeria*, *Pedobacter*, *Sphingobacterium*, *Edaphobaculum*, *Pajaroellobacter*, and *Weissella* were significantly more abundant in overwintering individuals (Figure 7C). Both permutated multivariate ANOVA (Adonis) and pairwise comparison of PERMANOVA revealed a strong segregation between the pre-overwintering and overwintering populations (all $p < 0.001$; Figure 7D).

Discussion

Here, we present a comprehensive assessment of different factors influencing the bacterial community composition of the European spruce bark beetle *I. typographus* across a wide geographic range including the four postglacial refugial areas. Moreover, we specifically investigated possible changes of the microbial community among populations in different epidemiological phases and populations before and during overwintering in an area with a current bark beetle mass outbreak within the Dolomites.

While previous studies were based on pooled insect guts from one restricted geographic area (Chakraborty et al. 2020; Fang et al. 2020; Veselská et al. 2022; Yu et al. 2022), our approach allowed a fine-scale analysis of several European populations on a single individual level. Our analysis revealed that the two genera *Erwinia* and *Pseudoxanthomonas*, which were previously described in *I. typographus* in the Czech Republic and in China (Chakraborty et al. 2020; Fang et al. 2020; Veselská et al. 2022; Yu et al. 2022), are present in all individuals analyzed across different regions, as well as epidemiological and overwintering phases. The omnipresence of these two genera across all individuals from different populations across Europe suggests that these bacteria are essential for *I. typographus* to successfully colonize trees and utilize the subcortical material as a food resource. Since various other bark beetle species of the genera *Ips* (Chakraborty et al. 2020) and *Dendroctonus* (Adams et al. 2013;

Durrant et al. 2015; Dohet et al. 2016) also harbored these bacteria, they might play an important role in the life histories of bark beetles. Strains of *Erwinia typographi* isolated from *I. typographus* were found to be tolerant to high concentrations of the monoterpene myrcene, one of the defensive compounds of Norway spruce (Skrodenytė-Arbačiauskienė et al. 2012), whereas members of the genus *Pseudoxanthomonas* contribute to cellulose and lignocellulose degradation and therefore play a potential role in the breakdown of plant cells in conifers (Kumar et al. 2015).

Apart from these two prevalent genera, other bacterial taxa were present in different populations, but at different frequencies. Since not all individuals harbor them, they are likely not essential for the survival of the host but might play an additional important role for the beetle. For example, *Brevundimonas* and *Pseudomonas*, have been shown to contribute in detoxification of conifer phytochemicals in *Dendroctonus* (Boone et al. 2013; Xu et al. 2015). While *Brevundimonas* is able to reduce diterpene abietic acid levels at low concentrations, two *Pseudomonas* species were shown to reduce concentrations of monoterpenes under controlled conditions (Boone et al. 2013). Since both taxa were present in all investigated populations, we assume that these bacterial taxa might be involved in overcoming host defenses and might therefore be especially important in colonizing healthy trees. Moreover, the genomic characterization of *Pseudomonas* strains isolated from *I. typographus* revealed various important pathways involved in the inhibition of entomopathogenic fungi as well as pathways for the hydrolyzation of cellulose, xylan, starch, and pectin (Peral-Aranega et al. 2020). Additionally, other dominant bacteria common among *Ips* species have been shown to be important for the biology of bark beetles: *Sphingobacterium* contributes to the decomposition of hemicellulose (Zhou et al. 2009) whereas *Acinetobacter* and *Williamsia* to the decomposition of lignin (Bugg et al. 2011). While *Sphingobacterium* and *Williamsia* were

present in most individuals from all different localities, *Acinetobacter* was present in lower abundance in fewer individuals from most populations.

Glacial refugial areas during the Pleistocene have been shown to be major evolutionary drivers in many species (Hewitt 2000; Schmitt 2007). While most studies focused on how the glacial isolation of different populations shaped their population structure (Bertheau et al. 2013; Schebeck et al. 2018), the potential effect on the microbial community has not been investigated yet. Here, we compared the bacterial communities of populations from the main putative refugial areas of *I. typographus* from the Apennine Alps (Abetone), the Dinaric Alps (Croatia), the Carpathian Alps (Rumania) and the Russian Plain (Stauffer et al. 1999; Bertheau et al. 2013), and observed significantly different bacterial communities between these populations. While the populations from the Apennines and Dinaric Alps clustered with the populations from Eastern Tyrol and South Tyrol, the populations from the Carpathian Mountains clustered with the populations from Veneto. This finding indicates a potential influence of the Carpathian refugial area onto the Southern Alps, which is different from the genetic structure of *I. typographus* (Stauffer et al. 1999, Bertheau et al. 2013; Papek et al. in revision) and therefore sheds new light on the evolution and post-glacial history of this bark beetle and its associated bacteria.

Although we found significant differences between the microbial communities from different regions, we did not detect major differences between the bacterial communities of populations in different epidemiological phases, neither in terms of taxonomic richness and diversity nor community structure. These results suggest that *I. typographus* outbreaks might not be linked to a major shift in the bacterial community, at least not in the early phase of an outbreak. However, a few changes were observed for specific genera among the most abundant bacteria, such as *Pseudoxanthomonas*, which had a higher relative abundance in the epidemic populations than in the endemic ones. These bacteria might influence the aggressiveness of the

insect in the epidemic phase and might help them to overcome host defenses, but this hypothesis remains to be investigated. Moreover, the genus *Spiroplasma* was also more abundant in the epidemic populations. Members of this genus are predominantly maternally inherited bacteria known to infect several arthropod and plant species (Duron et al. 2008). *Spiroplasma* have a diverse array of effects on their hosts, ranging from beneficial (such as increasing tolerance to natural enemies in their host) (Ballinger & Perlman 2019) to reproductive parasitism, particularly male-killing, where male host embryos are killed (Pool et al. 2006). The possible mutualistic or antagonistic relationships of the genus *Spiroplasma* with *I. typographus* and other bark beetles have, however, not yet been identified. Finally, the genus *Tyzzerella* was present in all studied localities and was more abundant in the endemic populations. Although *Tyzzerella* was detected in various spruce and pine beetle species (Chakraborty et al. 2020), its potential role in these species has yet to be investigated.

An additional important factor in the biology of *I. typographus* is the overwintering behavior. *I. typographus* increases its cold tolerance and enters a reproductive diapause at the end of the season to increase the chance of overwinter survival (Annila 1969; Schebeck et al. 2017; Schebeck et al. 2022). Seasonal variations and diapause behavior might influence the bacterial community structure and the latter could support the insect in overcoming the harsh winter conditions, for instance by nutrient storage to build up energy reserves. A change in the bacterial community structure across the season and during overwintering was observed in *Dendroctonus* bark beetles (Wang et al. 2017; Hou et al. 2021). By comparing populations sampled before and during the overwintering phase, we found a significant difference of the microbial community. In particular, the genera *Spiroplasma* and *Tyzzerella* were found at higher abundances in pre-overwintering populations, while *Chryseobacterium*, *Pseudomonas* and *Sphingobacterium* were more abundant in overwintering populations. The last three genera were also enriched during winter in the gut microbiota of *D. armandi* larvae (Wang et al. 2017),

highlighting a potential contribution in overwintering of bark beetles. It has been hypothesized that bacteria from the family *Sphingobacteriaceae* are associated with insect overwintering and survival at low temperatures (Wang et al. 2017), whereas *Pseudomonas* might be involved in increasing *I. typographus* resistance to fungal pathogens and thus increasing the chance of survival (Peral-Aranega et al. 2020). Additionally, the genus *Pedobacter* was significantly more abundant in overwintering populations. Although its role in bark beetles is currently not known, this genus also increased during diapause in *Colaphellus bowringi* (Chrysomelidae) (Liu et al. 2016).

In conclusion, our study on the bacterial community of *I. typographus* across different geographic regions and epidemiological and seasonal variations highlights the important role of bacteria in this important pest species. Especially the presence of *Erwinia* and *Pseudoxanthomonas* in all analyzed individuals suggests their essential role, likely as obligatory symbionts for this bark beetle. Further studies are needed to investigate their influence on the host and transmission route to the offspring. Similarly, the role of several less-frequent genera associated with *I. typographus* needs to be further investigated to understand the complex pattern across the life history of the European spruce bark beetle.

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Author contributions

HS, MS, CS, MF and AB designed the study, acquired funding, and performed the insect sampling. AM, PEV, GRS, EC, JD performed the lab work and data analysis, and AM, SN and HS wrote the manuscript with the contribution of all authors.

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Tables and Figures

Figure 1: Overview of *Ips typographus* populations. ET1-4: Eastern Tyrol; ST1-3: South Tyrol; VT1-6: Veneto; Abe: Abetone; CR: Croatia; RO: Romania and RU: Russia. Populations depicted in orange represent populations in endemic phase, whereas red indicates epidemic phase. Populations in green represent populations sampled before overwintering, whereas populations in blue were sampled during overwintering. The dashed background shows the area affected by the current *I. typographus* outbreak in the Southern Alps. Details of population localities are given in Table S1.

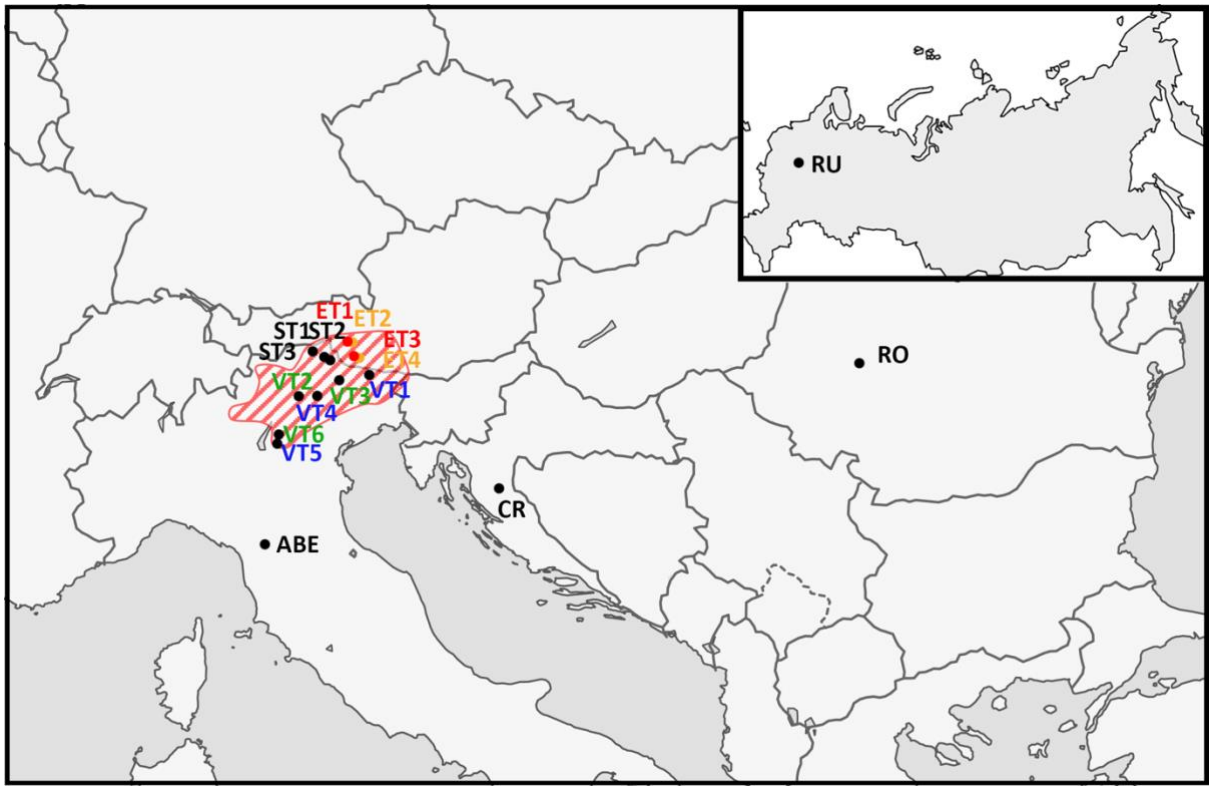

Figure 2: Microbial composition of *Ips typographus* across different populations at phylum level (A) and genus level (B). In (B) only the 15 most abundant OTUs were plotted.

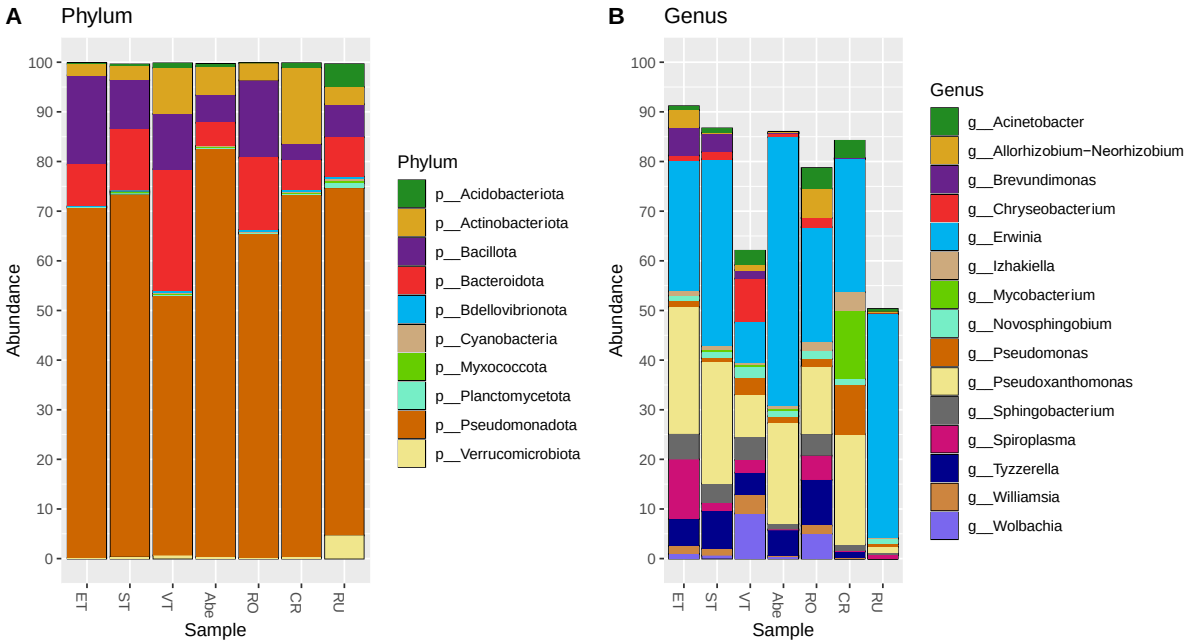

Figure 3: Distribution of OTUs across different populations of *Ips typographus*. Vertical bars (black) and the dot matrix represent the number of shared or unique OTUs, whereas horizontal bars (yellow) show the total number of OTUs present in each population.

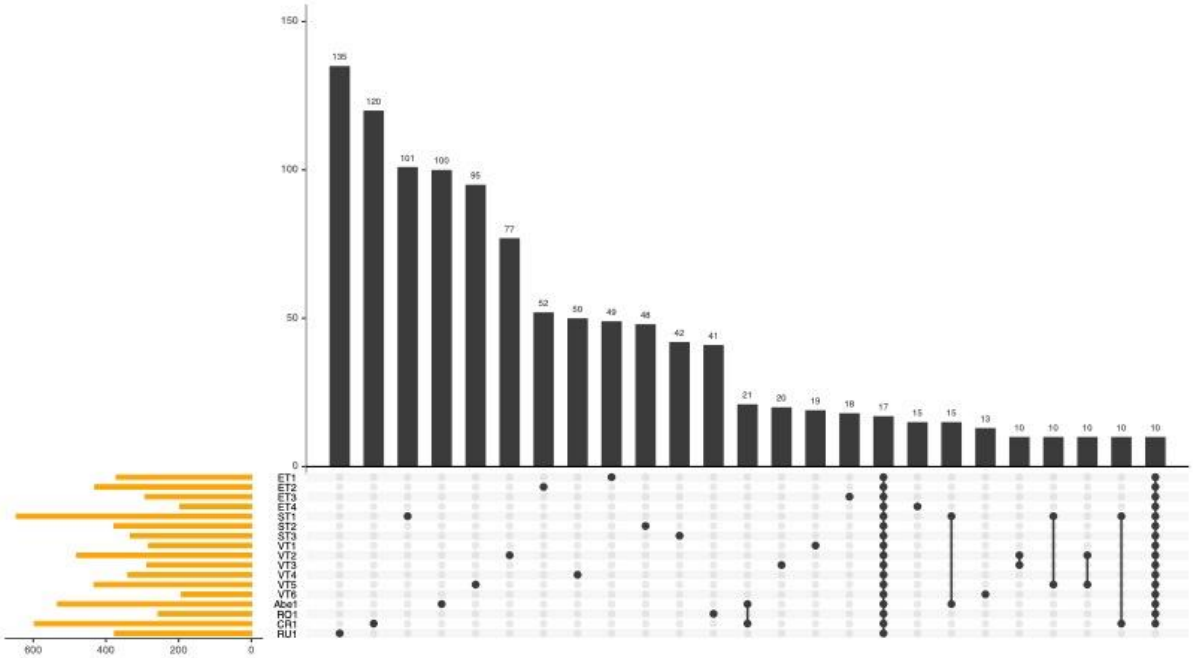

Figure 4: Alpha diversity of the bacterial communities of *Ips typographus* from different geographical regions. Comparison of bacterial species richness (Chao1, left) and bacterial diversity (Shannon index, right).

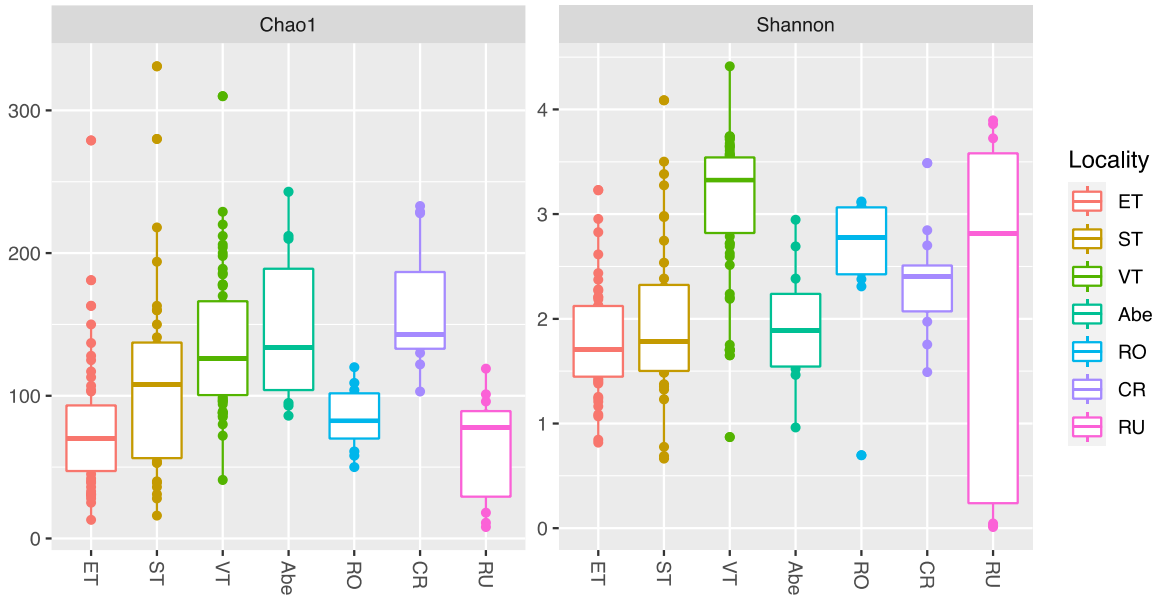

Figure 5: Canonical analysis of principal coordinates (CAP) (A) and hierarchical clustering (B) based on Bray-Curtis dissimilarity showing differences in bacterial community structure of *Ips typographus* between different geographic locations.

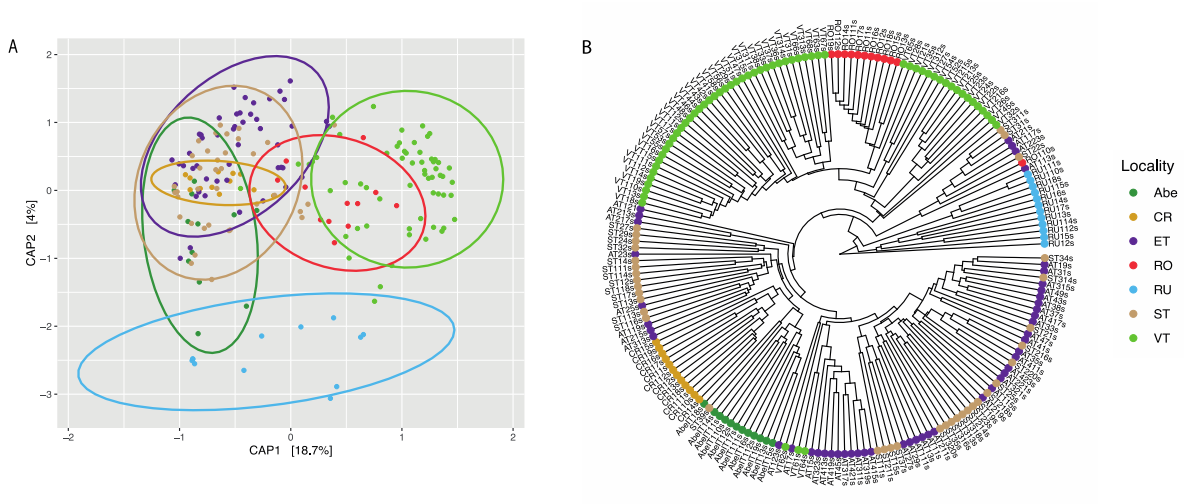

Figure 6: Microbial composition of endemic and epidemic populations of *I. typographus* from Eastern Tyrol at phylum level (A) and genus level (B). In (B) only the 15 most abundant OTUs were plotted. (C) Canonical analysis of principal coordinates (CAP) based on Bray-Curtis dissimilarity between endemic and epidemic populations.

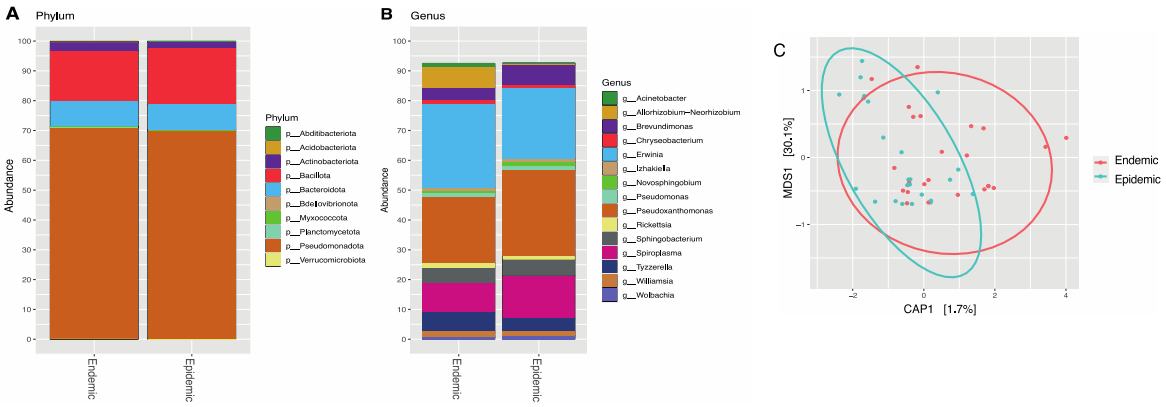
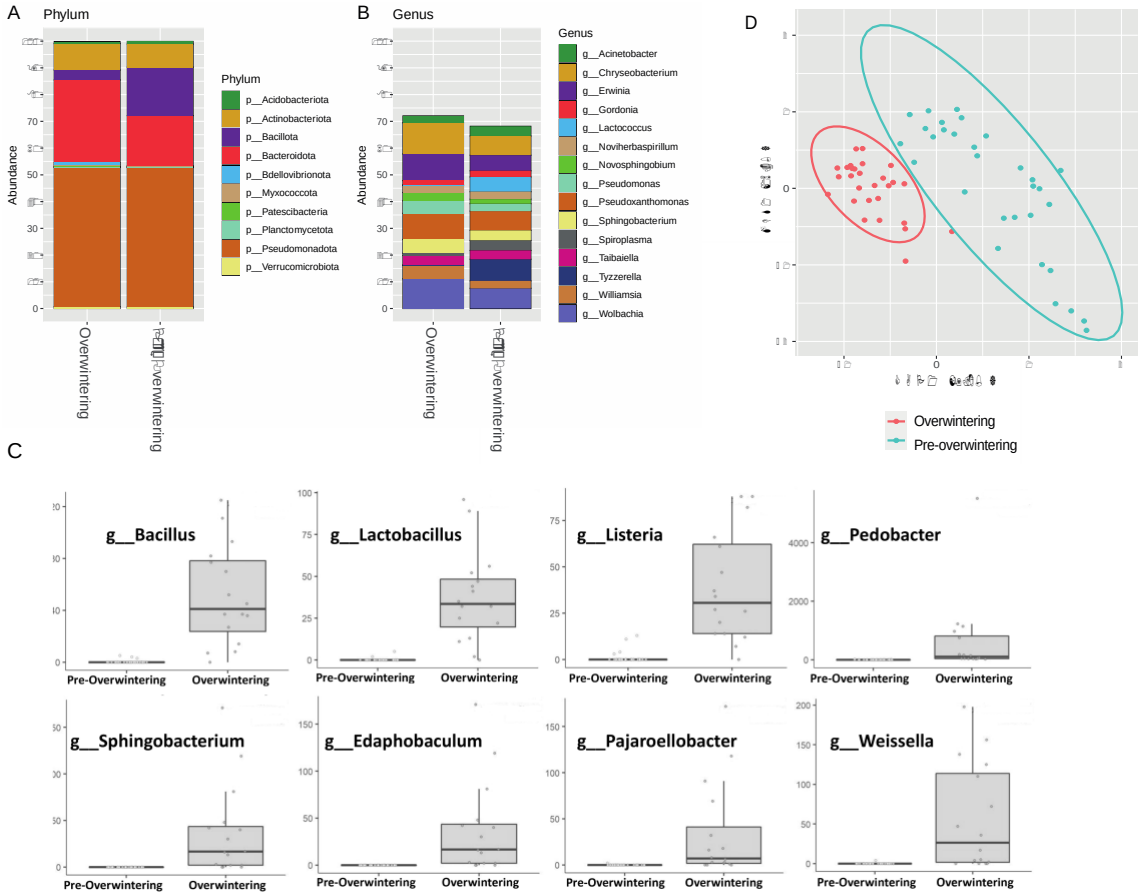
Figure 7: Microbial composition of pre-overwintering and overwintering populations of *I. typographus* from Veneto at phylum level (A) and genus level (B). In (B) only the 15 most abundant OTUs were plotted. (C) Differentially abundant taxa in pre-overwintering and overwintering populations from Veneto (D) and Canonical analysis of principal coordinates (CAP) based on Bray-Curtis dissimilarity between pre-overwintering and overwintering populations.