Ann. For. Res. 65(1): 97-110, 2022
https://doi.org/10.15287/afr.2022.2063

ANNALS OF FOREST RESEARCH
www.afrjournal.org

Next Generation Sequencing genomic analysis of bacteria from soils of the sites with naturally-occurring summer truffle (*Tuber aestivum* Vittad.)

Marta Siebyła[1], Dorota Hilszczańska[2]

Siebyła M., Dorota Hilszczanska D., 2022. Next Generation Sequencing genomic analysis of bacteria from soils of the sites with naturally-occurring summer truffle (*Tuber aestivum* Vittad.). Ann. For. Res. 65(1): 97-110.

Abstract The rhizosphere is the region of soil in which the highest densities of fungi and bacteria occur. In this study, an attempt was made to assess the distribution of bacterial species in soil where the summer truffle *Tuber aestivum* Vittad. bears fruit in selected stands in Poland. In order to determine the bacterial metagenome, the Next Generation Sequencing (NGS) method was applied. Differences occurred in the bacterial species composition at the cluster level between soils in which summer truffle fruiting was recorded and control soils. In particular, differences in the percentage of *Firmicutes* bacteria were noted with an average frequency of 3.9% in truffle soil compared to 96.1% in the control soil. It was estimated that two bacterial species, namely *Lysobacter antibioticus* and *Ensifer adhaerens* had a positive effect on the occurrence of *T. aestivum*. Our research increased the knowledge of particular groups of bacteria accompanying truffles and their potential impact on the formation of fruiting bodies in *T. aestivum*.

Keywords: soil bacteria, summer truffle, NGS method, bacterial diversity.

Addresses: [1]Department of Scientific Information and Promotion, Forest Research Institute, Raszyn, Poland| [2]Department of Forest Ecology, Forest Research Institute, Raszyn, Poland.

Corresponding Author: Marta Siebyła (m.siebyla@ibles.waw.pl)

Manuscript: received October 10, 2020; revised March 07, 2022; accepted June 21, 2022.

Introduction

The plant root zone is the soil layer most frequently inhabited by fungi and bacteria. It is estimated that the number of bacteria in the rhizosphere layer is one hundred times higher than outside the rhizosphere and amounts to about 2×10^9 cells per gram of soil and up to 4.8×10^9 in forest soils (Torsvik & Ovreas 2002). In this layer *Pseudomonas* bacteria are most common, as well as soil fungi, including those forming mycorrhiza, and they all interact directly with the plant root system (Badura 2004). The soil is also inhabited by underground ectomycorrhizal fungi commonly known as truffles, of the genus *Tuber*. Truffles are commonly found in various European countries including Germany, France, Italy and Spain. Previous studies have confirmed the occurrence of fruiting bodies of several valued species also in Poland, including *T. aestivum* Vittad., *T. macrosporum* Vittad., *T. mesentericum* Vittad., *T. borchii* Vittad. (Hilszczańska et al. 2013, Hilszczańska 2016, Hilszczańska et al. 2019 a, b), and *T.*

bellonae Quèl. (Ławrynowicz et al. 2008), however, the species has not been verified by molecular analyzes. Other species, which have no culinary properties, have also been found, including *T. rufum* Pico, *T. excavatum* Vittad., *T. maculatum* Vittad., and *T. fulgens* Quél. (Hilszczańska et al. 2013, Hilszczańska 2016, Hilszczańska et al. 2019 a, b).

In Poland, the best conditions for fruiting for the summer truffle are found in oak, beech and luminous oak forests. Hilszczańska et al. (2016) found that truffles are located in the areas where there are rendzina and pararendzina soils overlaying gypsum and marl. Truffle fruits are formed at a depth of about 10 – 20 cm, within the rhizosphere layer of forest trees and shrubs, which is inhabited by various microorganisms. Many factors influence truffle yield, including bacteria, yeasts, fungi and viruses in addition to suitable soil and weather conditions (Sbrana et al. 2002, Zacchi et al. 2003, Napoli et al. 2008, Gryndler & Hršelová 2012, Vahdatzadeh et al. 2015). As shown by Gryndler et al. (2012) and Vahdatzadeh et al. (2015), soil microorganisms colonize truffles at all stages of development, although their role in the ontogenesis of this fungi has not been fully explained. On one hand, they influence the nature of the truffle mycorrhizal compounds, while on the other, they are subject to the influence of the soil environment (Garbaye et al. 1992, Barbieri et al. 2005, Gryndler & Hršelová 2012, Siebyła et al. 2021).

Previous studies (Mamoun & Olivier 1992, Sbrana et al. 2002) showed that soil bacteria of the genera *Pseudomonas* and *Actinobacteria* and the family Bradyrhizobiaceae play beneficial roles in the development of truffle fruiting bodies. Gryndler et al. (2012) and others described the high probability of a link between summer truffle and some *Actinobacteria* species of the Pseudonocardineae family (genera: *Lentzea, Kibdelosporangium*) and Proteobacteria (*Lysobacter antibioticus*). Studies on the identification and importance of soil bacteria associated with the development and fruiting of summer truffle in Poland have not yet been conducted.

As less than 1% of soil microorganisms can be identified by traditional techniques using indirect methods, e.g. standard plate count or biochemical method (Schloss & Handelsman 2003), modern molecular analyses using metagenomics are currently applied (Itoh et al. 2014, Pacwa-Płociniczak et al. 2016, Jenkins et al. 2017). In this way, the metagenome of a particular soil environment can be studied (Courtois et al. 2003, Rajendhran & Gunasekaran 2008, Demanèche et al. 2009). However, the DNA extraction kits have some limitations which narrow the potential effectiveness of the assessment of community variability (Carrig et al. 2007, Morales et al. 2008). Knowledge of soil microbiological diversity is therefore still insufficient, regardless of species identification methods. The need for DNA extraction of all soil microbial communities in order to know the metagenome remains an issue. The underlying difficulty is due to the limitations of an extraction protocol that allows the isolation of only a part of the microbial population, adversely affecting the extraction of others. This issue is complicated by the great diversity of DNA in the soil. Research by Delmont et al. (2011) showed that biodiversity, depending on the applied DNA isolation method, is significant, ranging from 100 000 species (Roesch et al. 2007, Torsvik & Ovreas 2002), to 100 000 000 species (Gans et al. 2005) per gram of soil. Nevertheless, it allows the estimation of the characteristics of an identifiable population.

The aim of this study was to use the Next Generation Sequencing (NGS) method in order to determine the metagenome of bacteria occurring in forest soils in which the summer truffle *T. aestivum* bears fruit in selected stands in Poland. This was the basis for assessing the biodiversity measures of bacterial groups in the examined soils and their ecological position in the community, thus enabling us to determine the role of particular bacterial groups in the development of fruiting bodies of summer truffle, depending on stand dominant tree species.

Materials and Methods

Research areas

This study was carried out on three research sites in the area of the Nida Basin, marked as G, M and W, in which forest divisions with summer truffle (marked as T) and divisions without truffle (marked as C) were located (Siebyła & Hilszczańska 2020). The truffle and control locations were determined based on previous research by Professor Hilszczańska's team. The research sites are located at an altitude of 250 to 296 m above sea level and are situated in mixed forests that grow on rendzina type soils (State Forests Database (https://www.bdl.lasy.gov.pl/portal/mapy) (Forests Database Bank). For more information on geomorphology, climate and a map with the exact coordinates of the study area, see Siebyła et al. 2020. The dominant tree species in the studied stands are: pedunculate oak *Quercus robur* L. and hornbeam *Carpinus betulus* L. (areas G, M, W); small-leaved lime tree *Tilia cordata* Mill. and beech *Fagus sylvatica* L. (area M); and sycamore maple *Acer pseudoplatanus* L. (area W). In spring 2017, at each study site the mulch layer was removed to a depth of 10 cm, and a soil sample of approximately 4 dm^3 was taken with a shovel. Soil samples were collected from plots where the presence or absence of truffle ascomata had previously been detected (Hilszczańska et al. 2014). Three samples were taken in each site, G1-G3, M1-M3, W1-W3 for samples with summer truffles and G4-G6, M4-M6, W4-W6 for control samples. The soil samples were stored at -20°C until analyses commenced.

Metagenomic analysis of DNA samples

In order to isolate DNA, 0.5 g from each soil sample was weighed (3 repetitions per sample) and then instructions included in the Genomic Mini AX Soil Spin (A&A Biotechnology) isolation kit were followed (Przemieniecki et al. 2016). The 150 µl DNA isolated from each soil sample was stored at -20°C and then subjected to NGS and metagenome analysis based on the 16S RNA fragment (bacterial component). PCR products were sequenced by Genomed Joint-Stock Company, Warsaw, Poland (Janssen et al. 2002). Amplicon sequencing included a V3-V4 (or V4) fragment of the 16S rRNA gene enabling analysis of both Bacteria and Archaea taxonomic groups. Amplicons were prepared on samples of isolated DNA by PCR and 16S libraries were made. Sequencing was carried out using Illumina's MiSeq device in paired-end mode in two readings of 250 bases. For details on Illumina MiSeq sequencing, see Pacwa-Płociniczak (2020). The expected average number of reading pairs per sample was 100 000. Bioinformatic analysis included reading filtering and sample composition analysis for individual taxonomic categories based on database homologations (Medinger et al. 2010, Staley et al. 2013). Analysis was carried out in the Department of Microbiology at the Faculty of Biology and Environmental Protection of the University of Silesia using Qiagen (Hilden, Germany) equipment.

Genetic and biodiversity indicators

The genetic and biodiversity indicators were determined on the basis of: (a) alpha biodiversity of the community samples (either individual communities of forest soil within a defined delimited area or a collective list of species occurring within a particular geographical unit) and (b) beta diversity, which represents the diversity of species when comparing forest soil community changes in the environmental gradient.

Alpha biodiversity analysis

The alpha biodiversity indicator values were assessed based on the variability of identified Operational Taxonomic Unit (OTU) sequences (observed OTUs, number of distinct features) within a biological forest soil sample. The sample was defined by multiple biodiversity indices, including Chao, Faith's (Faith's phylogenetic diversity), OTUs observed, Heip's (Heip's evenness measure), and Shannon's and Simpson's indices (Shnnon 1948, Simpson 1949,

Heip 1974, Chao 1984, Faith 1992, Bolyen et al. 2019). Depending on the biodiversity index used, the value applied in the Kruskal-Wallis statistical test was calculated for all studied groups together and separately. The zero hypothesis assumes that all studied groups have an equal number of species. The calculated probability value of $p < 0.05$ rejects the null hypothesis. Chao, Faith's, OTUs, Shannon, Heip's, and Simpson indices were calculated using Qiime2 software (Bolyen et al. 2019, Monaco et al. 2020, Herrero de Aza et al. 2022) and implemented programs diversity alpha and diversity alpha group significance. All samples from the experiment, divided into individual groups described above, were used for analysis. Rarefaction charts and Kruskal-Wallis tests were also generated as a result of each of the analyses. The indices of species diversity were intended to show the individual differences resulting from the local conditions of truffle cultivation (habitat / population).

Chao index

The Chao index is the species abundance estimator and is determined by the non-parametric method of estimating the number of species in a microbiome. This index is focused on low abundance species, i.e. singletons and doubletons in the sample (Chao 1984). A singleton is defined as an OTU with exactly one sequence in the sample. A doubleton is defined as an OTU with exactly two sequences in the sample. This indicator is particularly useful for data sets that tend towards low-abundance species.

$$SChao = Sobs + (F1 \times (F1-1) / (2 \times (F2+1))$$

where: Sobs - number of species observed; F1, F2 - number of singletons and doubletons.

Faith's Phylogenetic Diversity Index

Phylogenetic diversity is a measure of biodiversity based on phylogenetic analysis. Faith (1992) defined the phylogenetic diversity of a microbiome as the sum of the lengths of all branches in a tree, encompassing a certain collection. If no members of a particular species (a given OTU) have been identified in one tree, the PD value of that tree is lower in comparison to the rest of the trees. Differences in PD values are an indication of which trees are more abundant than the others.

Observed Operational Taxonomic Units

Operational Taxonomic Units observed is an index that defines the number of unique OTU sequences in each sample (Bolyen et al. 2019).

Shannon index

The Shannon Index is the most commonly used biodiversity indicator. It takes into account both abundance and evenness of species present in the sample (Shannon 1948).

$$H = -\sum_{i=1}^{s} p_i \ln p_i$$

where: H - index; S - total number of species (OTU) in the microbiome; p_i - proportion of species (OTU) to the total number of species (OTU) in the Shannon microbiome.

Heip's Index

The Heip's index determines the species evenness index (OTU) between samples analyzed. The Shannon index value is used in the calculation. Compared to other indices, it is best suited for the analysis of low diversity microbiomes (Heip 1974).

$$E = (e^{H}-1)/(S-1)$$

where: H - the Shannon diversity index; S - species number.

Simpson Index

The Simpson diversity index is a measure of diversity that takes into account the number of species present (OTU) and their relative abundance. As the number and evenness of species increases, so does the diversity of the community. The value of the Simpson index is determined in a range of 0 to 1, where 1 indicates infinite diversity and 0 indicates no diversity (Simpson 1949).

$$D = 1 - (\Sigma n(n-1) / N(N-1))$$

where: *D* - Simpson index; *n* - total number of organisms per species; *N* - total number of organisms for all species.

Beta biodiversity analysis

Beta biodiversity is the variety of identified OTU sequences within a group of biological samples. It is defined by many biodiversity indicators such as unweighted and weighted unifrac, Bray-Curtis dissimilarity, Jaccard and others. All of these metrics were used to compare the identified microbiome between groups of forest soils samples. Statistical analysis of beta group significance results was performed using Qiime2 software (Bolyen et al. 2018) and the implemented diversity beta group significance program. The analysis used the PERMANOVA test, which identifies differences in distance between pairs of groups (Beals 1984, Lozupone & Knight 2005, Team 2013).

Unifrac

UniFrac is a measure of β diversity that uses phylogenetic data to compare environmental samples. UniFrac, combined with standard multidimensional statistical techniques, including main coordinate analysis (PCoA), is used to identify factors that account for differences between microbiological communities. UniFrac facilitates the understanding of the relationship between bacterial microbiomes and environmental diagnostics. It measures the difference between two sequential collections (for example, 16S rRNA particles derived from various microbiological samples) as a fraction of branch length in a phylogenetic tree. UniFrac allows for testing the relevance of phylogenetic line differences between samples, or for clustering multiple samples using multidimensional statistical techniques. The UniFrac weighted measure takes into account the occurrence of a given OTU, while the unweighted measure focuses on its' presence in the cluster. The PCoA (Principal component Analysis) charts resulting from the analyses performed with the R program show the distances between individual sample groups (Heip 1974).

Bray-Curtis Index

The Bray-Curtis Index, otherwise known as the dissimilarity index, is a measure used to assess the quantitative composition differences between individual groups.
This indicator is defined by the formula:

$$BC_{ij} = 1- (2C_{ij}/(S_i + S_j))$$

where: Bcij - Bray-Curtis index; C_{ij} - sum of the lowest frequencies for the same species (OTU) in groups S_i; S_i, S_j - total number of species (OTU) in both groups.

The value of the Bray-Curtis index lies in the range 0-1, with a value of 0 indicating that both groups have the same composition and a value of 1 indicating that the groups do not share a single species. This index is not a distance measure and should not be considered as a distance matrix between sample groups. It is a measure of the dissimilarity between samples, referred to as semimetric (Beals 1984).

Jaccard Index

The Jaccard similarity coefficient defines the common and different elements in a collection (sample groups). It is a measure of similarity between two groups of samples (in our case T and C), ranging from 0-100%. The higher the value of the Jaccard index the greater the similarity between two groups of samples. It is a very sensitive tool for cases of small numbers of analyzed samples within a group (Jaccard 2003).

Statistical analysis of beta-group-significance results was performed using Qiime2 software (Bolyen et al. 2019) and the implemented diversity beta-group-significance program. In the analysis, the PERMANOVA test was used, which identifies the distance differences between pairs of groups (Beals 1984, Lozupone & Knight 2005, Team 2013).

Results

Metagenomic analysis of DNA samples

Metagenomic analysis of DNA samples isolated directly from the soil (Figure 1) allowed us to

distinguish six dominant classes of bacteria: Proteobacteria (30–65%), Actinobacteria (7–36%), Bacteroidetes (1–30%), Acidobacteria (0.3–30%), Planctomycetes (0.2–10%), and Chloroflexi (2–23%). Bacteria from classes: Chloroflexi and Actinobaceria were more common in the soils where summer truffle was found (~60%), while Proteobacteria and Firmicutes prevailed in the control soils (< 15%).

The NGS sequencing of soil samples resulted in an average of 648.00 to 1 017.00 taxonomic units (OTUs) in areas where truffle fruiting bodies were recorded and 532.33 to 788.00 OTUs in the control soil which had no truffles (Table 1).

The value of the Chao index on all three plots (M, G and W) was on average higher (2 634.84) in comparison with the control soil (2 179.9). Results of the Faith, Shannon, Heip, and Simpson diversity indices were similar regardless of the soil (Table1).

Table 1 Alpha-biodiversity assessment using Chao, Faith, OTUs, Shannon, Heip, and Simpson diversity indices in soil samples from Nida Basin sites M, G, and W.

S	Alpha biodiversity					
	OTU	Chao	Faith	Shannon	Heip	Simpson
MT	841.33	941.78	37.04	7.94	0.30	0.99
MC	788.00	819.23	36.68	7.99	0.34	0.99
GT	648.00	656.45	31.79	7.41	0.27	0.98
GC	532.33	597.71	26.15	7.21	0.29	0.98
WT	1017.00	1036.61	44.44	8.67	0.40	0.99
WC	691.00	762.96	30.39	7.94	0.37	0.99

Notes: S- Sample; T- soils where *T. aestivum* was present, C - control soil, where *T aestivum* was absent.

Variability of the samples (calculated using the coefficient of variation v%) varied for individual indicators. The coefficient of variation for OTU and Chao Faith, Shannon's, and Chao Faith indices in control samples G and W was more than twice as high as in those samples where truffle fruiting bodies were found. On area M the difference between T and C sites was less significant.

For Simpson's index the results were very consistent. The highest value of V% = 1.44 was recorded in sample GT. Sample G (Simpson's index), regardless of the soil, showed a half greater value of the coefficient of variation

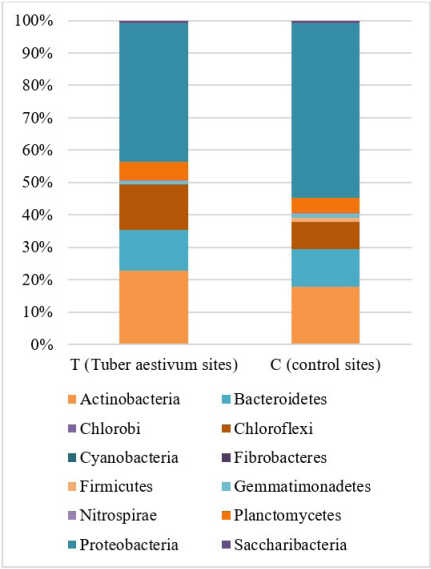

Figure 1 Average percentage share of the 12 most numerous bacterial phyla in soil samples of the study sites T (sites where *T. aestivum* was present) and C (control sites, where *T. aestivum* was absent).

Table 2 Values of coefficient of variation v (%) of biodiversity for the soils with and without *T. aestivum* presence.

S	Indices					
	OTU	Chao	Faith	Shannon	Heip	Simpson
MT	21.06	26.85	10.52	4.35	5.15	0.59
MC	29.95	31.66	20.25	6.76	19.78	0.59
GT	13.75	13.93	9.56	2.96	29.35	1.44
GC	30.44	32.97	13.53	8.50	21.53	1.02
WT	12.50	13.44	2.95	2.46	2.50	0.58
WC	45.43	53.19	29.20	7.66	5.66	0.00

Notes: S- Sample; T- soils where *T. aestivum* was present, C - control soil, where *T aestivum* was absent.

compared to samples M and W. Low values were recorded for the Shannon index. The coefficient of variation v% for Shannon index in samples G and W were twice as high in soil C as in soil T. Differences between the soils may indicate the local impact of the microhabitat on high diversity of communities even for a single sample (Table 2).

Assessment of genetic and biological diversity

Chao index

Statistical analysis using the Kruskal-Wallis test for all groups together showed no differences

in the number of singletons and doubletons between sample groups. The comparison of individual samples demonstrated a difference in microbial biodiversity between groups of samples, in terms of the amount of OTU of small size for the group of samples GT vs WT ($p = 0.08$, $p < 0.1$), and GC vs WT ($p = 0.04$, $p < 0.05$) (Figure 2). For particular groups of samples the microbiomes do not change above a certain threshold. For GT samples the value is 2 000 readings and for GC samples it is 10 000 readings.

Faith's index

The statistical analysis of Kruskal-Wallis showed differences in PD values significant for all groups ($p = 0.07$, $p < 0.1$). Additionally, differences in PD trees were observed between GC vs MT ($p = 0.049$, $p < 0.5$), GC vs WT ($p = 0.049$, $p < 0.5$), GT vs WT ($p = 0.08$, $p < 0.1$), MC vs WT ($p = 0.049$, $p < 0.05$), MT vs WT ($p = 0.049$, $p < 0.05$) and WC vs WT ($p = 0.08$, $p < 0.1$) (Figure 3).

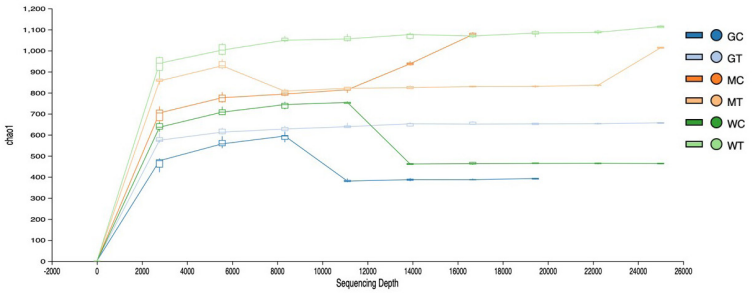

Figure 2 Chao index rarefaction curves showing results for soils with (GT, MT, WT) and without (GC, MC, WC) *T. aestivum*.

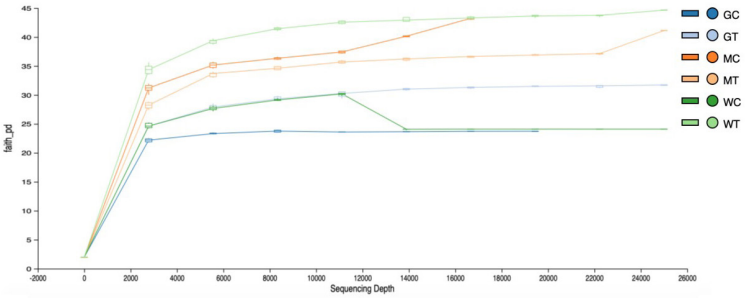

Figure 3 Faith's index rarefaction curves showing results for soils with (GT, MT, WT) and without (GC, MC, WC) *T. aestivum*.

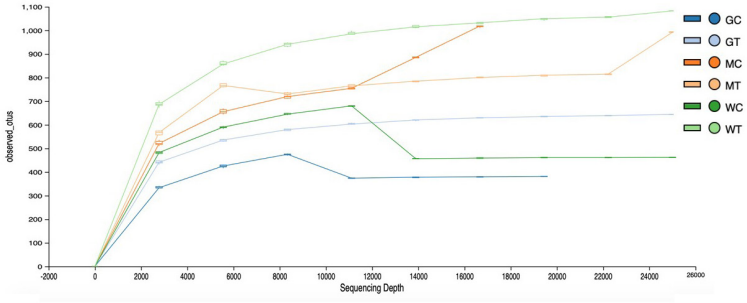

Figure 4 Operational Taxonomic Unit index rarefaction curves showing results for soils with (GT, MT, WT) and without (GC, MC, WC) *T. aestivum*.

Observed OTUs

Kruskal-Wallis analysis for all groups together did not show any differences in OTU counts between sample groups. Statistical analysis between individual sample groups showed the difference between GT vs WT ($p = 0.08$, $p < 0.1$), and GC vs WT ($p = 0.04$, $p < 0.05$) (Figure 4).

Shannon index

The Kruskal-Wallis analysis proved the differences in Shannon values to be statistically significant for all study groups ($p = 0.08$, $p < 0.1$).

Differences were observed between GC vs WT ($p = 0.049$, $p < 0.05$), GT vs MC ($p = 0.08$, $p < 0.1$), GT vs MT ($p = 0.08$, $p < 0.1$), GT vs WT ($p = 0.08$, $p < 0.1$), MT vs WT ($p = 0.049$, $p < 0.05$) and WC vs WT ($p = 0.08$, $p < 0.1$) (Figure 5).

Heip's index

Kruskal-Wallis analysis showed differences in Heip's values for all study groups ($p = 0.06$, $p < 0.1$). Differences were observed between GC vs WC ($p = 0.08$, $p < 0.1$), GT vs WT ($p = 0.049$, $p < 0.05$), GT vs WT ($p = 0.08$, $p < 0.1$), MC vs W T ($p = 0.049$, $p < 0.05$), MT vs WC ($p = 0.08$, $p < 0.1$) and MT vs WT ($p = 0.049$, $p < 0.05$) and WC vs WT ($p = 0.08$, $p < 0.1$) (Figure 6).

Simpson's Index

Kruskal-Wallis statistical analysis for all groups together found no differences in Simpson values between sample groups. Statistical analysis between individual samples showed differences between GT vs WT ($p = 0.08$, $p < 0.1$), and MT vs WT ($p = 0.049$, $p < 0.05$).

Beta biodiversity analysis

Unifrac

Both the weighted and the non-weighted measures of the occurrence of a given OTU showed a distribution of diversity among the sample groups. The control samples GC and WC differed the least, and showed much less similarity to the other sample groups. Statistical analysis using the biodiversity indicators unweighted and weighted Unifrac indicated significant differences in phylogenetic lines between GC and WC samples and the other samples. A similar pattern is observed in the samples MC and MT (unweighted measure) and MC, MT and WT (weighted measure) (Figure 7 and 8). Figure 8 shows that the GT samples are significantly distant from the others, as are the WT samples.

The PERMANOVA test for all the examined groups revealed statistical differences at the level of probability $p = 0.001$, so the differences in distances and indicators in the analyzed groups are significant. The prepared boxplot charts show the differences in distances between the analyzed groups. The value n is determined by the number of comparisons.

Bray-Curtis index

The PCoA graph, which includes the values derived from Bray-Curtis index analysis, clearly shows

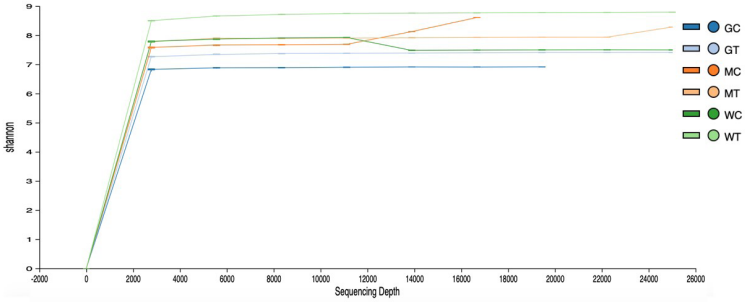

Figure 5 Shannon Index rarefaction curves showing results for soils with (GT, MT, WT) and without (GC, MC, WC) *T. aestivum*.

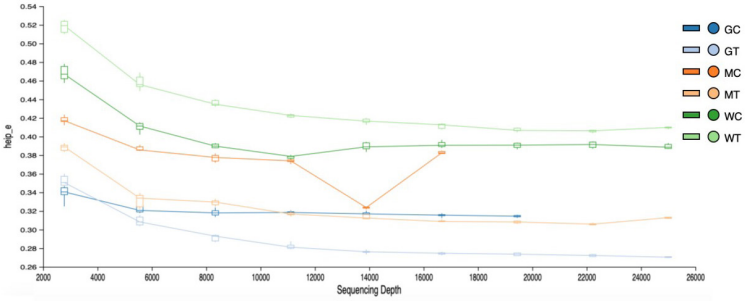

Figure 6 Heip's Index rarefaction curves showing results for soils with (GT, MT, WT) and without (GC, MC, WC) *T. aestivum*.

the divisions between sample groups. The WC and GC control samples (similarly to UniFrac measurement) showed a small similarity of bacterial communities, as was shown in Figure 7 and 8 but were different from other groups. The samples WT and MT and MC were similar to each other, but significantly different from the others. Only the GT samples revealed a large difference in composition compared to the previous groups (Figure 9).

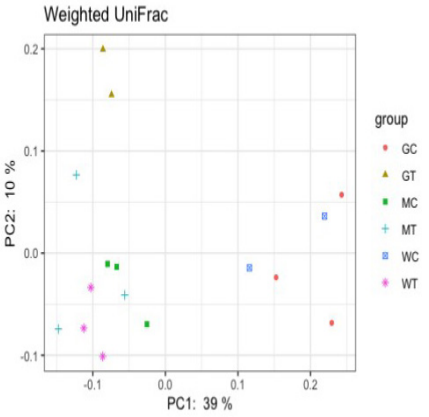

Figure 7 Analysis of Operational Taxonomic Unit bacterial coordinates of UNIFRAC weighted ranges showing results for soils with (GT, MT, WT) and without (GC, MC, WC) *T. aestivum*.

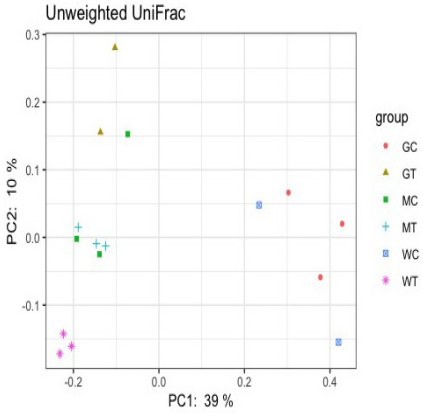

Figure 8 Analysis of Operational Taxonomic Unit bacterial coordinates of UNIFRAC unweighted ranges unweighted ranges showing results for soils with (GT, MT, WT) and without (GC, MC, WC) *T. aestivum swapped charts 8 and 9 (8 instead of 9 and 9 with 8, like in the word draft)*.

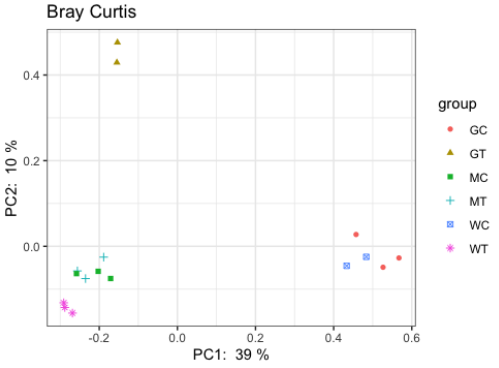

Figure 9 Bray-Curtis index PCoA distance graph showing the results for soils with (GT, MT, WT) and without (GC, MC, WC) *T. aestivum swapped charts 8 with 9, 9 with 8.*

Jaccard index

The PCoA graph for Jaccard values shows significant differences between sample groups. Similarly to the UniFrac and Bray-Curtis measures, the WC and GC samples showed little resemblance to each other, but differed significantly from the other groups. The MC and MT groups were shown to be similar to each other, but were significantly different from the others. The WT group is clearly distinct from the rest of the study groups, as is the GT group (Figure 10).

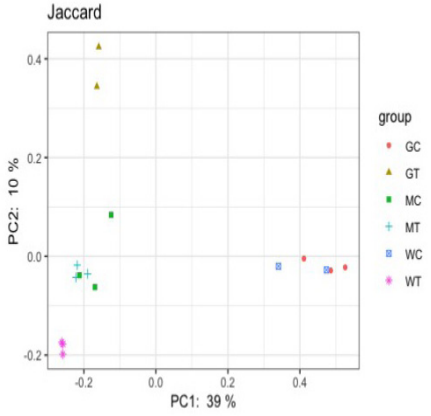

Figure 10 Jaccard index PCoA distance graph showing the results for soils with (GT, MT, WT) and without (GC, MC, WC) *T. aestivum*.

Discussion

The results of NGS sequencing demonstrated that the dominant bacterial populations in the soil samples from the Nida Basin area were bacteria belonging to the Proteobacteria and Actinobaceria classes. However, comparative results between the two variants showed differences in the number of bacteria. Bacteria from the classes: Chloroflexi and Actinobaceria were more numerous in the variant with summer truffle (~ 60%), while Proteobacteria and Firmicutes dominated in the control variant (< 15%). Moreover, NGS sequencing of soil samples revealed an average of 648.00 to 1017.00 taxonomic units (OTU) in the plots where truffle fruiting bodies were detected and 532.33 - 788.00 OTU in the control variant without truffles. Similar results for bacterial diversity were obtained by Gryndler et al. (2013) and Mello et al. (2013), who compared the diversity of bacteria inhabiting the soil in which *T. aestivum* was recorded with control samples where the bacterium was absent. Soil samples tested by Gryndler et al. (2013) were collected in the suburbs of Prague (Velká Chuchle) in mixed deciduous forest where hornbeam (*C. betulus*) was the dominant species and soil pH ranged from pH 6–7 as in our study. Research conducted by Hilszczańska et al. (2019a, b) on areas G and W were located in stands not subject to human impact and composed of different species, although with similar soil pH to those described by Gryndler et al. (2013) (pH 6.9 and 7.5 respectively).

The research by Siebyła et al. (2020) and previous studies by Hilszczańska et al. (2019a, 2019b) showed that the influence of soil chemical composition on the occurrence of truffle fruiting bodies is of great importance. The soil was mainly determined by the content of calcium ions. The percentage (%) of calcium ions was almost twice as high in the truffle variant (42.6 cmol / kg on average), compared to the control variant (24.06 cmol / kg). Similarly, N, C, K were almost twice as high in the samples where truffle fruiting bodies were detected compared to the control variant in the soils of sites G and W. Plots G and W were located in stands that were not influenced by humans and consisted of other forest vegetation, and soil pH (pH 6.9 and 7.5, respectively) was similar (Hilszczańska et al. 2019a, 2019b) as described by Gryndler et al. (2013). Plots M and W were dominated by upland forest and fresh forest, respectively. Plot W was characterized by the dominance of tree species such as *Q. petrea* (85 years), A. pseudoplatanus and *C. betulus*, while plot M was dominated by *Q. robur* (140 years), *T. cordata*, *C. betulus* and *F. sylvatica*. In plot G, *C. betulus* (100 years) and *Q. robur* species were predominant. The mentioned sites differed not only in terms of soil chemistry, but also in terms of vegetation, which was reflected in the differences in the total number of bacteria and actinomycetes in the soil. Siebyła et al. (2020) described that the total number of bacteria in truffle soils averaged 6.6 log 10 cfu and in control soils 6.3 log 10 cfu. For actinomycetes, the average bacterial count in truffle soils was 6.0 log 10 cfu and in control soils was 5.6 log 10 cfu.

The study by Mello et al. (2013) showed the influence of bacterial communities on the development of *Tuber melanosporum* by the PhyloChip method in France. The dominant tree species was *Quercus pubescens* Willd. (truffle plantation de Cahors-Le Montat), and the dominant classes of bacteria were Proteobacteria, Actinobaceria and Firmicutes. In soil samples taken in the Nida Basin, NGS sequencing indicated that the Firmicutes class is not a dominant phylogenetic group here. The results obtained in this study on the Firmicutes class are in agreement with Gryndler et al. (2013), in a study conducted using the sequencing of 454 soil samples taken at sites where *T. aestivum* was recorded along with control samples. In the study by Mello et al. (2013), a general pyrosequence-based analysis of bacterial communities showed that the dominant classes were Proteobacteria (15 to 17%), Actinobacteria (17 to 20%), Bacteroidetes (8 to 10%), Firmicutes (1 to 5%), Verrucomicrobia (1 to 5%), Acidobacteria

(0.2 to 4%), Planctomycetes (0.4 to 2%), Chloroflexi (0.3 to 1%) and Chlamydiae (0.2 to 1%) (Deveau et al. 2016). These results indicate a significant impact of microbial communities on the soil environment.

The use of advanced metagenetics is necessary to investigate interactions between bacteria in soil ecosystems. According to Gryndler et al. (2013) and Deveau et al. (2016) some members of the family *Pseudonocardineae* (class Actinobacteria) were associated with ectomycorrhizal truffles. Representatives of this family were among the bacteria isolated in this work, although they were not as numerous as in Gryndler et al. (2013). Results reported by Deveau et al. (2016) indicate that bacteria of the family *Pseudonocardineae* (order Actinomycetes) were abundant in the ectomycorrhizal fungi *T. aestivum* and *T. melanosporum* in samples taken as early as January, in the garden of Rollainville (Lorraine region, France), where the dominant species is the common hazel (*Corylus avellana* L.).

Occurrence of genera of *Bradyrhizobium* and *Rhizobium* in the samples collected in the Nida Basin was quite variable in all three locations *Bradyrhizobium* sp. 89S1MB was recorded only on the MT and WK areas and *Rhizobium* sp. rf050 on the G and W control areas. Gryndler et al. (2013) listed the genus *Bradyrhizobium* (order *Rhizobiales*) as being negatively correlated with the summer truffle, while Barbieri et al. (2005) and Cerigini et al. (2008) reported positive associations of these bacteria, except for the presence of *T. borchii*.

Streptomyces bacteria were found in all samples where summer truffle fruiting was reported, and also in the control. However, some species, i.e.: *Streptomyces xanthocidicus* (area W), *Streptomyces scabrisporus* (M), *Streptomyces* sp. old-30-2-1 (M), *Streptomyces* sp. P617A (W), *Streptomyces* sp. UYEF31 (M) were found only in soil where summer truffle fruiting bodies were recorded, which may indicate their direct association with these fungi. These results also correspond to reports by Frey-Klett et al. (2007) on some representatives of the genera *Rhodococcus, Streptomyces* and *Arthrobacter* as typical bacterial species for the development of ectomycorias of truffles and other fungi. The species *Lysobacter antibioticus* was recorded only on the area M of the truffle soils, as described by Gryndler et al. (2013) in the Czech Republic, while strain *Ensifer* sp. Bmb17 was present only on area G in the truffle soils. According to Gryndler et al. (2013) *Lysobacter antibioticus* and *Ensifer adhaerens* species positively correlate with the occurrence of *T. aestivum,* which is in agreement with results obtained in this study. Bacteria of the genus *Lysobacter* exhibit antagonistic properties against microorganisms such as *Phytophthora* sp. and pathogenic fungi and nematodes (Ko et al. 2009). *Lysobacter antibioticus* is a bacterial species of the PGPR (plant growth-promoting rhizobacteria), which promotes the colonization of host roots and thus inhibits the pathogen by producing antibiotics, lytic enzymes, volatile compounds and siderophores. This may directly or indirectly protect plants against pathogenic attack (Ko et al. 2009). Research conducted by Kobayashi et al. (2009) proved that the enzymes of the *Lysobacter* genus are capable of colonizing mycelia both internally and externally.

Bacteria of the genus *Ensifer* also exhibit antagonistic properties. These microorganisms help reduce salt stress in plants by decreasing Na uptake (Sandhya et al. 2010). The *E. adhaerens* TMX-23 strain produces salicylic acid, which is an important phytohormone associated with plant defence reactions to biotic stress (Zhou et al. 2013). The *E. adhaerens* bacterium produces the antibiotic allicin, which is also an antifungal agent. Studies carried out by Yutani et al. (2010) showed that allicin demonstrates an antifungal effect on *Saccharomyces cerevisiae* and the pathogenic yeast *Candia albicans*. In turn, the genus *Kibdelosporangium*, which was described by the abovementioned authors as being positively correlated with the occurrence of summer truffle, was present

in the Nida Basin only in control soils (M and G areas). The genus *Lentzea* was recorded in the truffle soil in area G and in the control soil in area W, which indicates that the soils in these stands were colonized randomly.

The use of new identification techniques seems indispensable when defining the role of bacteria in relation to fungal growth, for example the *Pseudonocardineae* bacteria associated with the genus *Tuber*. This study is the first attempt to use NGS sequencing to learn about the metagenome of bacteria isolated from soils with a confirmed truffle presence. This analysis enabled us to distinguish bacterial communities present in soils where truffles are present, which consequently broadens the knowledge of how to control bacterial communities in truffle plantations (Garbaye et al. 1992). This is of great importance in the case of *T. aestivum*, the most common truffle species, in Poland which has recently been recently cultivated as a crop (Hilszczańska et al. 2013).

In this study, the alpha and beta biodiversity analysis showed differences between the control soil samples and the "truffle" soil samples. The results obtained showed a clear separation between the two groups. Jenkins et al.'s (2017) studies of microbiome change and soil function using next-generation sequencing also showed differences between the control and treatment samples. Beta diversity, the similarity between taxa identities and their abundances after treatment, was assessed using pairwise UNIFRAC distances (similar to this study). Site beta diversity revealed significant differences in bacterial community structure and abundance. The studies conducted also used weighted UNIFRAC and unweighted UNIFRAC.

Conclusion

A significant and positive relationship was found between the occurrence of *Lysobacter antibioticus* and *Ensifer adhaerens* species in the soil and the presence of fruiting bodies of summer truffle *T. aestivum*.

A negative correlation between the occurrence of bacteria of the genus *Bradyrhizobium* (order *Rhizobiales*) in the soil and the presence of summer truffle fruiting bodies was found.

Streptomyces (family *Actinomycetaceae*) were present in all samples regardless of the soil, which is a proof of their ubiquitous character.

Species and strains of *Streptomyces xanthocidicus*, *Streptomyces scabrisporus*, *Streptomyces* sp. old-30-2-1, *Streptomyces* sp. P617A, *Streptomyces* sp. UYEF31 were recorded only in soil where summer truffle fruiting bodies were present, which may be of monitoring importance.

There were significant differences in the proportion of *Firmicutes* bacteria depending on the soil type - in truffle soil their frequency was 3.9% on average and 96.1% in the control soil.

Greater homogeneity of bacterial communities representing truffle soils was found in comparison with those from control soils, which was confirmed by more than twice lower values of the coefficient of variation v% for OTU, Chao Faith, Shannon, Heip's and Simpson's indices.

The alpha (Faith's, Shannon, Heip's) and beta (weighted and unweighted measures, Bray-Curtis, Jaccard) biodiversity indicators used have proven useful in assessing the diversity of bacterial communities in the soil samples studied.

Acknowledgements

The research was carried out with the funds of the statutory activities of the Forest Research Institute, issue no. 240326/900326. I would like to thank the employees of the Department of Microbiology of the Faculty of Biology and Environmental Protection of the University of Silesia for their invaluable comments and guidance during the research. I would like to thank too Mrs Agata Młodzińska for the help and for performing bioinformatics analysis.

References

Badura L., 2004. Mikroorganizmy glebowe i ich znaczenie w ekosystemach degradowanych przez człowieka. Inżynieria ekologiczna 12: 14-15.

Barbieri E., Bertini L., Rossi I., Ceccaroli P., Saltarelli R., Guidi C., Zambonelli A., Stocchi V., 2005. New evidence for bacterial diversity in the ascoma of the ectomycorrhizal fungus *Tuber borchii*. FEMS Microbiology Letters 247: 23–35. https://doi.org/10.1016/j.femsle.2005.04.027

Beals E.W., 1984. Bray-Curtis ordination: an effective strategy for analysis of multivariate ecological data. Advances in

Ecological Research 14.
Bolyen E., Rideout J.R., Dillon M.R., Bokulich N.A., Abnet C.C., Al-Ghalith G.A., ... & Caporaso J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology, 37(8), 852-857. https://doi.org/10.1038/s41587-019-0209-9
Carrig C., Rice O., Kavanagh S., Collins G., O'Flaherty V., 2007. DNA extraction method affects microbial community profiles from soils and sediment. Applied Microbiology and Biotechnology 77:955–964. https://doi.org/10.1007/s00253-007-1219-y
Cerigini E., Palma F., Barbieri E., Buffalini M., Stocchi V., 2008. The *Tuber borchii* fruiting body-specific protein TBF-1, a novel lectin which interacts with associated *Rhizobium* species. FEMS Microbiology Letters 284(2): 197-203. https://doi.org/10.1111/j.1574-6968.2008.01197.x
Chao A., 1984. Nonparametric estimation of the number of classes in a population. Scandinavian Journal of statistics: 265-270.
Courtois S., Cappellano C.M., Ball M., Francou F.X., Normand P., Helynck G., Martinez A., Kolvek S.J., Hopke J., Osburne M.S., August P.R., Nalin R., Guérineau M., Jeannin P., Simonet P., Pernodet J.L., 2003. Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. Applied and Environmental Microbiology 69: 49-55. https://doi.org/10.1128/AEM.69.1.49-55.2003
Delmont T.O., Robe P., Cecillon S., Clark I.M., Constancias F., Simonet P., Hirsch P.R., Vogel T.M., 2011. Accessing the soil metagenome for studies of microbial diversity. Applied and Environmental Microbiology 77(4): 1315-1324. https://doi.org/10.1128/AEM.01526-10
Demanèche S., Philippot L., David M.M., Navarro E., Vogel T.M., Simonet P., 2009. Characterization of denitrification gene clusters of soil bacteria via a metagenomic approach. Applied and Environmental Microbiology 75(2): 534-537. https://doi.org/10.1128/AEM.01706-08
Deveau A., Antony-Babu S., Le Tacon F., Robin C., Frey-Klett P., Uroz S., 2016. Temporal changes of bacterial communities in the *Tuber melanosporum* ectomycorrhizosphere during ascocarp development. Mycorrhiza 26(5): 389-399. https://doi.org/10.1007/s00572-015-0679-7
Faith D.P., 1992. Conservation evaluation and phylogenetic diversity. Biological Conservation 61(1): 1-10.
Forests Database Bank (https://www.bdl.lasy.gov.pl/portal/mapy).
Frey-Klett P., Garbaye J.A., Tarkka M., 2007. The mycorrhiza helper bacteria revisited. New Phytologist 176(1): 22-36. https://doi.org/10.1111/j.1469-8137.2007.02191.x
Gans J., Wolinsky M., Dunbar J., 2005. Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science 309(5739): 1387-1390. https://doi.org/10.1126/science.1112665
Garbaye J., Churin J.L., Duponnois R., 1992. Effects of substrate sterilization, fungicide treatment, nd mycorrhization helper bacteria on ectomycorrhizal formation of pedunculate oak (*Quercus robur*) inoculated with *Laccaria laccata* in two peat bare-root nurseries. Biology Fertility Soils 13 (1): 55-57.
Gryndler M., Hršelová H., 2012. Isolation of bacteria from ectomycorrhizae of *Tuber aestivum* Vittad. Acta Mycologica 47 (2): 155-160. https://doi.org/10.5586/am.2012.018
Gryndler M., Soukupová L., Hršelová H., Gryndlerová H., Borovička J., Streiblová E., Jansa J., 2013. A quest for indigenous truffle helper prokaryotes. Environmental Microbiology Reports 5 (3): 346-352. https://doi.org/10.1111/1758-2229.12014
Heip C., 1974. A new index measuring evenness. Journal of the Marine Biological Association of the UK 54(3): 555-557.
Herrero de Aza C., Armenteros S., McDermott J., Mauceri S., Olaizola J., Hernández-Rodríguez M., & Mediavilla O., 2022. Fungal and bacterial communities in *Tuber melanosporum* plantations from Northern Spain. Forests, 13(3): 385. https://doi.org/10.3390/f13030385
Hilszczańska D., Rosa-Gruszecka A., Sikora K., Szmidla H. 2013. First report of *Tuber macrosporum* occurrence in Poland. Scientific Research and Essays 8 (23): 1096-1099. https://doi.org/10.5897/SRE2013.5529
Hilszczanska D., Rosa-Gruszecka, A., Szmidla, H. 2014. Characteristic of *Tuber* spp. localities in natural stands with emphasis on plant species composition. Acta Mycologica, 49(2): 267-277.
Hilszczańska D., 2016. Polskie trufle skarb odzyskany. CILP, Warszawa (54).
Hilszczańska D., Szmidla H., Sikora K., Rosa-Gruszecka A. 2019a. Soil properties conducive to *Tuber aestivum* Vitt. fruiting bodies formation in the Nida Basin stands. Polish Journal of Environmental Studies 28(3): 1713-1718. https://doi.org/10.15244/pjoes/89588
Hilszczańska D., Rosa-Gruszecka A., Gawryś R., Horak J., 2019b. Effect of soil properties and vegetation characteristics in determining the frequency of Burgundy truffle fruiting bodies in Southern Poland. Ecoscience 26(2): 113-122. https://doi.org/10.1080/11956860.2018.1530327
Itoh H., Navarro R., Takeshita K., Tago K., Hayatsu M., Hori T., Kikuchi Y., 2014. Bacterial population succession and adaptation affected by insecticide application and soil spraying history. Frontiers in Microbiology 5:457. https://doi.org/10.3389/fmicb.2014.00457
Jaccard J., Turrisi R., 2003. Interaction effects in multiple regression. Thousand OKS, Sage 72, London (104).
Janssen P.H., Yates P.S., Grinton B.E., Taylor P.M., Sait M. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Applied and Environmental Microbiology, 68, 2391-2396.
Jenkins J.R., Viger M., Arnold E.C., Harris Z.M., Ventura M., Miglietta F., Girardin C., Edwards R.J., Rumpel C., Fornasier F., Zavalloni C., Tonon G., Alberti G., Taylor G., 2017. Biochar alters the soil microbiome and soil function: results of next-generation amplicon sequencing across Europe. Global Change Biology Bioenergy 9(3): 591-612. https://doi.org/10.1111/gcbb.12371
Ko H.S., Jin R.D., Krishnan H.B., Lee S.B., Kim K.Y., 2009. Biocontrol ability of *Lysobacter antibioticus* HS124 against *Phytophthora* blight is mediated by the production of 4-hydroxyphenylacetic acid and several lytic enzymes. Current Microbiology 59(6): 608-615. https://doi.org/10.1007/s00284-009-9481-0
Kobayashi D.Y., Crouch J.A., 2009. Bacterial/fungal interactions: from pathogens to mutualistic endosymbionts. Annual Review of Phytopathology 47: 63-82. https://doi.org/10.1146/annurev-phyto-080508-081729
Ławrynowicz M., Krzyszczyk T., Fałdziński M., 2008. Occur-

rence of black truffles in Poland. Acta Mycol. 43 (2): 143-151. https://doi.org/10.5586/am.2008.018

Lozupone C., Knight R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Applied and Environmental Microbiology 71(12): 8228-8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Mamoun M., Olivier J.M., 1992. Effect of soil Pseudomonads on colonization of hazel roots by the ecto-mycorrhizal species *Tuber melanosporum* and its competitors. Plant Soil 139: 265-273.

Medinger R., Nolte V., Pandey R.V., Jost S., Ottenwaelder B., Schloetterer C., Boenigk J., 2010. Diversity in a hidden world: potential and limitation of next-generation sequencing for surveys of molecular diversity of eukaryotic microorganisms. Molecular Ecology 19: 32-40. https://doi.org/10.1111/j.1365-294X.2009.04478.x

Mello A., Ding G.C., Piceno Y. M., Tom L.M., DeSantis T.Z., Andersen G.L., Smalla K., Bonfante P., 2013. Truffle brûlés have an impact on the diversity of soil bacterial communities. PLoS One 8(4): e61945. https://doi.org/10.1371/journal.pone.0061945

Monaco P., Toumi M., Sferra G., Tóth E., Naclerio G., & Bucci A., 2020. The bacterial communities of *Tuber aestivum*: preliminary investigations in Molise region, Southern Italy. Annals of Microbiology, 70(1): 37. https://doi.org/10.1186/s13213-020-01586-5

Morales S.E., Cosart T.F., Johnson J.V., Holben W.E., 2008. Extensive phylogenetic analysis of a soil bacterial community illustrates extreme taxon evenness and the effects of amplicon length, degree of coverage, and DNA fractionation on classification and ecological parameters. Applied and Environmental Microbiology 75: 668-675. https://doi.org/10.1128/AEM.01757-08

Napoli C., Mello A., Bonfante P., 2008. Dissecting the rhizosphere complexity: the truffle-ground study case. Rendiconti Lincei. Scienze Fisiche Naturali 19: 241-259. https://doi.org/10.1007/s12210-008-0017-5

Pacwa-Płociniczak M., Biniecka P., Bondarczuk K., Piotrowska-Seget Z., 2020. Metagenomic functional profiling reveals differences in bacterial composition and function during bioaugmentation of aged petroleum-contaminated soil. Frontiers in Microbiology 11:2106. https://doi.org/10.3389/fmicb.2020.02106

Przemieniecki S.W., Kurowski T.P., Kotlarz K., Krawczyk K., Damszel M., Karwowska A., 2016. Plant growth promoting properties of *Serratia fonticola* ART-8 and *Pseudomonas putida* ART-9 and their effect on the growth of spring wheat (*Triticum aestivum* L.). Environmental Biotechnology, 12 (2) 35-39.

Rajendhran J., Gunasekaran P., 2008. Strategies for accessing soil metagenome for desired applications. Biotechnology Advances 26: 576-590. https://doi.org/10.1016/j.biotechadv.2008.08.002

Roesch L., Fulthorpe R.R., Riva A., Casella G., Hadwin A.K.M., Kent A.D., Daroub S.H., Camargo F.A.O., Farmerie W.G., Triplett E.W., 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. ISME Journal 1: 283-290. https://doi.org/10.1038/ismej.2007.53

Rosa-Gruszecka A., Hilszczańska D., Szmidla H. 2014. Warunki środowiskowe sprzyjające występowaniu trufli (*Tuber* spp.) na historycznych stanowiskach w Polsce. Leśne Prace Badawcze 75 (1): 5-11.

Sandhya V.S.K.Z., Ali S.Z., Grover M., Reddy G., Venkateswarlu B., 2010. Effect of plant growth promoting *Pseudomonas* spp. on compatible solutes, antioxidant status and plant growth of maize under drought stress. Journal of Plant Growth Regulation 62(1): 21-30. https://doi.org/10.1007/s10725-010-9479-4

Sbrana C., Agnolucci M., Bedini S., Lepera A., Toffanin A., Giovannetti M., Nuti M.P., 2002. Diversity of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas and their activity on *T. borchii* mycelial growth. FEMS Microbiology Letters 211 (2): 195-202. https://doi.org/10.1111/j.1574-6968.2002.tb11224.x

Schloss P.D., Handelsman J., 2003. Biotechnological prospects from metagenomics. Current Opinion in Biotechnology 14(3): 303-310. https://doi.org/10.1016/S0958-1669(03)00067-3

Shannon C.E., 1948. A mathematical theory of communication. Bell Labs Technical Journal 27(3): 379-423.

Siebyła M., Hilszczańska D., 2020. Diversity of soil bacteria complexes associated with summer truffle (Tuber aestivum). Folia Forestalia Polonica, Series A – Forestry 62 (2), 114-127.

Siebyła M., Szyp-Borowska I., 2021. Comparison of bacterial communities in roots of selected trees with and without summer truffle (*Tuber aestivum*) ectomycorrhiza. Folia Forestalia Polonica, 63(2): 97-111. https://doi.org/10.2478/ffp-2021-0011

Simpson E.H., 1949. Measurement of diversity. Nature 163: 688.

Staley C., Unno T., Gould T.J., Jarvis B., Phillips J., Cotner J.B., Sadowsky M.J., 2013. Application of Illumina next-generation sequencing to characterize the bacterial community of the Upper Mississippi River. Journal of Applied Microbiology 115(5): 1147-1158. https://doi.org/10.1111/jam.12323

Team R.C., 2013. A language and environment for statistical computing. 1-1706.

Torsvik V., Ovreas L., 2002. Microbial diversity and function in soil: from genes to ecosystems. Current Opinion in Microbiology 5 (3): 240-245. https://doi.org/10.1016/S1369-5274(02)00324-7

Vahdatzadeh M., Deveau A., Splivallo R., 2015. The role of the microbiome of truffles in aroma formation: a meta-analysis approach. Applied and Environmental Microbiology 81: 6946-6952. https://doi.org/10.1128/AEM.01098-15

Yutani M., Taniguchi H., Borjihan H., Ogita A., Fujita K.I., Tanaka T., 2010. Alliinase from *Ensifer adhaerens* and its use for generation of fungicidal activity. AMB Express 1(1): 1-8. https://doi.org/10.1186/2191-0855-1-2

Zacchi L., Vaughan-Martini A., Angelini P., 2003. Yeast distribution in a truffle-field ecosystem. Annals of Microbiology 53: 275-282.

Zhou G.C., Wang Y., Zhai S., Ge F., Liu Z.H., Dai Y.J., Yuan S., Hou Y.J., 2013. Biodegradation of the neonicotinoid insecticide thiamethoxam by the nitrogen-fixing and plant-growth-promoting rhizobacterium *Ensifer adhaerens* strain TMX-23. Applied Microbiology and Biotechnology 97(9): 4065-4074. https://doi.org/10.1007/s00253-012-4638-3