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Diversity of soil bacteria complexes associated with summer truffle (*Tuber aestivum*)

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Abstract

This paper describes the quantitative and qualitative composition of bacteria isolated from soil in the selected sites in the Nida Basin, in places where mycorrhizae and ascocarps of summer truffle (*Tuber aestivum*) were found, and in a control soil (without truffle). A classic growth culture method was used with Sanger DNA sequencing to obtain quantitative and qualitative measures of bacterial cultures. The obtained results showed differences in bacteriome composition between the case samples, in which summer truffle fructification was observed, and the control samples. Seven classes of bacteria were identified: Actinobacteria, Bacilli, Deinococci, Flavobacteria, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria. The most numerous bacterial genera were *Pseudomonas* (class Gammaproteobacteria) – 33%, *Streptomyces* (class Actinobacteria) – 29% and *Bacillus* (class Bacilli) – 15%. This research broadens the understanding of individual groups of bacteria accompanying truffles and their potential impact on the formation of summer truffle ascocarps.

Key words

mycorrhiza, Sanger DNA sequencing, soil microorganisms

Introduction

Due to its physical properties and chemical composition, soil provides a suitable growth environment for numerous microbial species, particularly bacteria and fungi. The physicochemical composition of soil and vegetation determines the composition of organisms, while microbes, through their metabolites, may also have a direct or indirect influence on the habitat and vegetation. The quantitative and qualitative compositions of the soil microbiome depend on the type, structure and physicochemical factors of the soil, its moisture, pH, temperature, and nutrient content (Zwoliński 2005; Frąc and Jezierska-Tys 2010). The number of bacteria per gram of soil ranges from several hundred to ten to twenty thousand colony forming units (CFU). The bacterial count in 1 cm^3 of forest soil is approximately 4.8×10^9 CFU, for forest litter $7.5–9.5 \times 10^8$ and in waterlogged soil $10^9–10^{10}$ CFU (Torsvik and Ovreas 2002; Krivtsov et al. 2005; Ipsilantis and Sylvia 2007). Depending on environmental factors, the quantitative and qualitative composition of the bacterial community may change over time. Differences in species abundance can result from varying diversity of local populations, sometimes even within a single site.

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The soil layer inhabited by the greatest number of microbes is the root zone of plants, the rhizosphere. The number of bacteria in this zone is about 2×10^9 cells per gram and is up to a hundred times greater than that outside of the rhizosphere. The rhizosphere is dominated by gram-negative bacteria from the genus *Pseudomonas*, which directly cooperate with the root system. They have a beneficial effect on the plant, for example, by facilitating nitrogen uptake, supplying mineral components or producing pyoverdines responsible for inhibiting the growth of phytopathogens, thus increasing plant health (Pociejewska et al. 2014; Siebyła and Hilszczańska 2017).

Soil is also a habitat for fungi, including underground fungi from the truffle genus (*Tuber* spp.), which belong to the sac fungi (Ascomycota). These are present in many European countries, for example, France, Italy, Spain, as well as Poland. The representatives of the genus *Tuber* create ectomycorrhizae with many tree species, for example, oak, beech, lime, hornbeam, black pine, and aspen, and shrubs, for example, hazel (Baciarelli-Falini et al. 2006). Until recently, truffles were not well known in Poland and the occurrence of *T. aestivum* was questioned (Hilszczańska 2016). However, in the last few years, knowledge in this field has been significantly expanded. It has been confirmed that, in Poland, there are valuable edible truffle species, such as summer truffle (*T. aestivum*), smooth black truffle (*T. macrosporum* Vitt.), Lorraine truffle (*T. mesentericum* Vitt.) and white truffle (*T. borchii* Vitt.), as well as species with no culinary value, such as: red truffle (*T. rufum* Pico), hollowed truffle (*T. excavatum* Vitt.), speckled truffle (*T. maculatum* Vitt.) and bright truffle (*T. fulgens* Quél.) (Hilszczańska et al. 2013; Rosa-Gruszecka et al. 2014; Hilszczańska et al. 2019a, b).

The development of mycorrhizae, including those of different truffle species, like the growth of bacteria, is regulated by the temperature, humidity, structure and chemical parameters of the soil. Some bacteria are referred to as plant growth-promoting bacteria (PGPB) or 'helpers', as they can stimulate the mycorrhizal colonization of host roots, and consequently, affect the development of ascocarps, including those of truffles (Gryndler et al., 2013). Truffle ascocarps are formed in the soil at a depth of 10–20 cm, so it is likely that the microbes present in the rhizosphere layer influence the development of mycorrhiza and truffle mycelia developing in the soil (Garbaye et al. 1992; Barbieri et al. 2005).

Research by Hilszczańska et al. (2019 a) and Rosa-Gruszecka et al. (2014) showed that in Poland, summer truffle enjoys the best conditions for fructification in mixed stands, in habitats of broadleaved forests, beech forests, and bright oak trees. Truffle sites are located mainly in areas where there are rendzic (humus-calcareous soils) or pararendsinas soils formed on gypsums and marls with pH values of 7–8 units. Precipitation and temperature have a significant influence on truffle yield. However, the most influential factors in the growth of this type of fungi are the content of calcium and calcium carbonate in the soil and its structure (Hilszczańska 2016). Studies by Hilszczańska et al. (2019 a, b) concerned the physicochemical properties of soils in which ascocarps of summer truffles were found. They have demonstrated that the most favourable conditions for the development of this fungus are found in soils with a calcium content of 19.6–36.6 cmol/kg^{-1} and a calcium carbonate content of 0.26–36.56%. The soil structure also influences the development of truffle mycorrhizae. Optimum soil proportions include 6.69–46.00% sand, 25.22–52.00% silt and 24.29–55.40% clay.

At all stages of their life cycle, specimens of truffle (mycelium, mycorrhizae, ascocarps) are colonized by microbes, such as bacteria, yeasts, filamentous fungi and viruses (Baldrian et al. 2012; Vahdatzadeh et al. 2015). The occurrence of truffles is associated with specific populations of bacteria inhabiting the roots of plants that host the fungi from the genus *Tuber*. The outer and inner parts of truffle ascocarps contain bacteria in large numbers, which may range from one million million cells per gram of dry mass of the ascocarp (Barbieri et al. 2005; Barbieri et al. 2007; Olivier et al. 2012). Previous studies carried out in southern Europe show that soil bacteria of the genus *Pseudomonas*, the actinomycetes (type Actinobacteria) and Bradyrhizobiaceae play a beneficial role in the growth of ascocarps of *Tuber* spp. These bacteria protect host plants against drought, supply nitrogen to the mycelium forming the ascocarp and stimulate its growth (Citterio et al. 1995; Sbrana et al. 2002; Barbieri et al. 2007; Saltarelli et al. 2008; Barbieri et al. 2010). According to Gryndler et al. (2013) and Vahdatzadeh et al. (2015), the fungus *T. aestivum* is also associated with certain species of bacteria.

The objective of this study was to determine the diversity of bacterial communities occurring in the soil, in which the summer truffle *T. aestivum* fructifies, and bacterial communities in the soil without truffle (control soil), in selected stands of the Nida Basin. Current knowledge of the bacterial content of 'truffle' soils is limited, therefore, the presented results shed new light on typical biotic conditions for the growth of truffle mycelia and ascocarps in Poland.

Material and methods

Research plots

Soil samples were collected from three forest stands (marked G, M, and W) in the Nida Basin, the southern part of Poland, which is 342.27 Nida Basin Mesoregion (Fig. 1, after Solon et al. 2018). Research plots were located in previously localized allotments, in which summer truffle ascocarps were present (Hilszczańska et al. 2019a, b), marked as T (with truffle) and in allotments without truffles, marked as C (control). The research plot stands were situated at an altitude of 250–296 m above sea level and, according to the Forest Database, they are located in habitats classified as mixed forest (G), fresh forest (M) and upland forest (W), all with rendzic soils. The dominant tree species

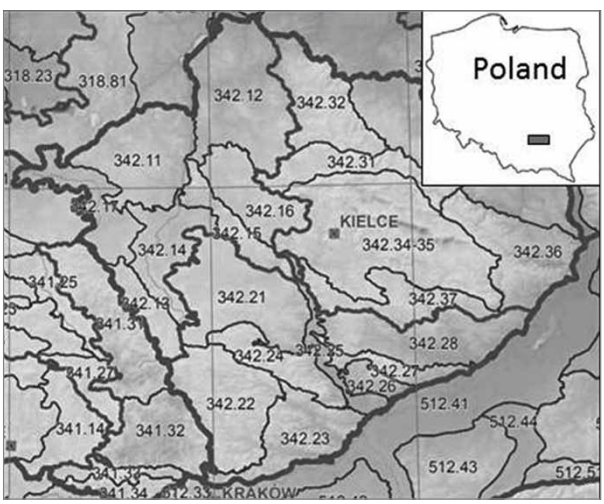

Figure 1. Map of the 342.27 Mesoregion of the Nida Basin. *Source:* Solon J. et al. (2018) with Geographia Polonica Journal 2019 permission. *Disclaimer*: Anyone who wishes to reuse the figure must respect the Creative Commons CC BY 4.0 license

on the research plots are pedunculate oak (*Quercus robur* L.) and hornbeam (*Carpinus betulus* L.), as well as small-leaved lime (*Tilia cordata* Mill.) and at lower frequency, beech (*Fagus silvatica* L.) (plot M only) and sycamore maple (*Acer pseudoplatanus* L.) (plot W only). In the plots where summer truffle fructified, the soil was described as rendzic; humus-calcareous soils (Hilszczańska 2016).

Chemical analysis of soil

Soil samples were collected for chemical analysis from variant plots on a one-off basis in spring 2017, from a depth of 10 cm after removal of the top layer of litter. A pooled sample was prepared from three sample 'iterations'. The physical structure of each soil matched the proposed optimal conditions for truffle growth (Hilszczańska et al. 2019a, b).

The chemical composition of each soil was analysed by the Laboratory of Environmental Chemistry of the Forest Research Institute, which holds AB 740 certification from the Polish Centre for Accreditation. The soil reaction with water and potassium chloride was determined using the PN-ISO 10390:1997 method. The percentage content of nitrogen was determined as per the PN-ISO 13878:2002 standard, and carbon as per the PN-ISO 10694:2002 standard. The carbon-nitrogen ratio, phosphorus oxide (V) P_2O_5 and calcium carbonate $CaCO_3$ content were established according to testing procedures PB-20 (2014) and PB-08 (2014). The content of exchangeable cations of Ca, K, Mg in soil was determined on the basis of the PN-ISO 11260:2011 standard.

Microbiological analysis of bacterial communities in soil

Soil samples for microbiological analysis were collected from the outlined research plots in spring and autumn 2016–2017. After the removal of an upper layer of litter, 18 soil samples (0.5 kg each) were collected from each quadrant following a factorial design, which totalled 72 samples (two years, two dates, three plots, 3 randomly taken samples from each truffle soil [T] and 3 samples from soil without truffle [C]). Soil samples were stored at –20°C until the analysis to limit microbial growth.

The compositions of bacterial communities were analysed using: i) classical and ii) molecular methods. Bacteria were cultured in solid media in 5 replications, then strains were identified by the following methods:

Quantitative analysis – classical method

From each soil sample, after mixing, 10 g was weighed out, placed in a volumetric flask filled with 0.85% NaCl solution to a volume of 100 ml and shaken for 15 minutes. Each sample was serially diluted from 10^{-2} to 10^{-4} in test-tubes, each containing 9 ml of 0.85% NaCl solution, using 1 ml of soil suspension from the previous dilution step. A bacterial suspension volume of 0.1 ml was poured into solid selective media with appropriate dilutions, in Petri dishes, this step was repeated five times. To determine the total number of bacteria in these samples, soil suspension (with dilution –4) was poured into a medium with nutrient agar (Gotkowska-Płachta et al. 2008). Fluorescent bacteria (dilution –2) were poured on to King's B medium (Gotkowska-Płachta et al. 2008) to identify *Pseudomonas fluorescens*. For the identification of actinomycetes, soil suspension (dilution –3) was poured into the Pochon medium (Gotkowska-Płachta et al. 2008). Samples were incubated in the dark at 37°C. Cultured bacterial colonies were counted after the incubation period, which for fluorescent bacteria was 12, 24 and 48 hours, for a total count of bacteria 4 days, and for actinomycetes 10 days. The base 10 logarithms of the colony count ($\log_{10}$ CFU) were calculated and averaged for each variant.

Qualitative analyses – molecular method

For qualitative analyses, material from a single bacterial colony was isolated from bacterial agar following growth in the liquid medium. DNA was extracted according to the suggested procedure for the Bacterial Genomic Miniprep Kit (from Sigma Aldrich). Isolated DNA was amplified through PCR using primers generic to bacteria: 530f (5'GTGCCAGCMGCCGCGG'3) and 1100R (5'GGGTTGCGCTCGTTG'3) (Lane 1991; Gryndler et al. 2012). The PCR thermal profile was as follows: 94°C for 4 min; 30 cycles at 94°C for 60 s, 62°C for 40 s, and 72°C for 2 min; and a final extension at 72°C for 6 min. Amplifications were carried out in 10 µl with 1 µl of DNA, 0,2U/µl Taq- polymerase (Qiagen), 1 µl 10x PCR buffer (Qiagen), 1.5 mM Mg (25 mM) (Qiagen), 0.1 mM dNTP (5 mM) (Qiagen), 0.1 µl of each primer (10 µM), 5 µl 25x Q buffer (Qiagen). Products were cleaned with the Clean-up Kit (from A&A Biotechnology). PCR products were sequenced by Genomed Joint-Stock Company, Warsaw, Poland (Janssen et al. 2002). Finch TV software was used to read the sequencing results. The obtained sequences were compared with the Gene Bank NCBI database using Blast (http://www.ncbi.nlm.nih.gov).

Meteorological data

Temperature and hydrological data for the nearest measuring stations Kielce and Kraków were counted on the basis of monthly Bulletins of the State Hydrological and Meteorological Service of the Institute of Meteorology and Water Management (IMGW-PIB). On this basis, Sielianinov's hydrothermal coefficient K for the years 2014–2017 was calculated ($K= P\times10/\Sigma t$), where P is the sum of precipitation and Σt is the sum of average temperatures during the vegetation period). Coefficient K values mean: 0–0.4, extremely dry; 0.41–0.70, very dry; 0.71–1.0, quite dry; 1.1–1.3, dry; 1.31–1.6, optimal; 1.61–2.0, moist; 2.1–2.5, wet; > 2.5, very wet.

Statistical analyses

To identify the differences in the physical soil composition among plots and variants, two-factor analyses of variance (ANOVA, normalized by $\log_{10}$) were performed, adjusting for repeated measurements using Tukey's post-hoc test. To identify the differences in bacterial count between variants (T and C), plots (G, M, W), and the evaluation period (spring, autumn), two-factor ANOVAs were performed, adjusting for repeated measurements using the post-hoc NIR test. Data were analysed in Statistica, version 10 (2011), applying a significance threshold of $p < 0.05$.

Results

Soil evaluation

The pH_{H2O} values of samples where fructification of summer truffles was observed ranged from 7.03 to 7.37. For control soils, pH_{H2O} values ranged from 4.93 to 7.07. N, C, K and Ca^{2+} ion compositions differed significantly between the control variant and the samples where summer truffle ascocarps were found on plots G and W, although, in the case of plot M, there were no differences in the relative proportions of N, C, K and ions Ca^{2+} between truffle and control soil samples.

The proportion of calcium ions was three times higher in truffle soils than in controls at plot W, and five times higher at plot G. However, in the case of

plot M, the results were similar for plots MT and MC (Tab. 1).

The ANOVA results show that, at all three plots, all soil chemical parameters differed between soils with confirmed presence of summer truffle and control soils, with the exception of phosphorous pentoxide (P_2O_5). For ions of P_2O_5, there were no differences between variants or areas.

Weather conditions

The weather conditions in two years preceding the first year of analysis were in particular months very variable – after a rather humid 2014 there was a drought, especially felt from June to August 2015 (Tab. 2). In 2015, in the spring-summer season, the range of Sielianinov's hydrothermal coefficient ranged from 0.17–0.82. Also in 2016, periods of drought were noted in May–June and September in Kielce station. The Sielianinov hydrothermal coefficient was 0.49–0.63 (Kielce station), and in autumn 2016 in August (0.86) and September (0.41) (Kraków station), respectively. June 2017 was extremely dry, which could have an impact on the activity of both bacterial communities and the fruiting of truffles.

In all evaluated years, October was full of rainfall, which was reflected in higher values of the hydrothermal coefficient.

Microbiological analyses

Traditional quantitative analysis

The estimated count of bacteria in soils where the summer truffle was found (the average for three plots, for all evaluation dates) was 6.6 $\log_{10}$ CFU, and, in soils with no truffle ascocarps, 6.3 $\log_{10}$ CFU. The quantitative composition of actinomycetes was 6.0 $\log_{10}$ CFU for samples from the areas with summer truffle, and 5.6 $\log_{10}$ CFU for the control samples. Despite significant differences in chemical composition, the average total share of bacteria was similar among the examined soils.

Table 1. Chemical composition of soil samples analysed and results of ANOVA (shaded)

Designation of samples	pH-KCl	pH-H_2O	N (%)	C (%)	C/N	$CaCO_3$ (%)	P_2O_5 (mg/100g)	Ca (cmol(+)/kg)	K (cmol(+)/kg)	Mg (cmol(+)/kg)
GT	6.50	7.03	0.37	4.17	11.23	0.38	3.71	33.20	0.87	1.22
GC	3.77	4.93	0.22	2.48	11.43	0.00	1.79	6.01	0.27	0.71
MT	6.67	7.17	0.45	6.34	14.13	0.84	3.76	45.36	0.57	1.16
MC	6.63	7.07	0.51	7.00	13.60	0.71	5.84	48.25	0.57	1.52
WT	7.00	7.37	0.60	8.33	13.87	9.52	4.71	49.22	0.94	1.32
WC	4.97	5.73	0.30	3.89	13.33	1.84	3.91	17.68	0.24	0.59
Area	0.021	0.045	0.033	0.006	0.008	0.001	ns	0.005	ns	ns
Variant	0.001	0.002	0.037	0.029	0.637	0.010	ns	0.005	0.000	ns
Interaction	0.033	ns	0.051	0.047	ns	0.007	ns	0.047	0.002	0.029

ns – not significant.

Table 2. Mean hydrothermic coefficient K for IV–X months at the nearest measuring stations Kielce and Kraków in 2014–2017. Values < 1.0 bold marked

Station name	IV	V	VI	VII	VIII	IX	X	K	Years
Kielce	1.18	3.06	1.50	2.65	1.72	0.78	1.34	1.75	2014
Kraków	1.23	2.64	1.27	1.22	1.68	1.48	1.07	1.51	
Kielce	1.17	2.82	1.24	0.82	0.17	1.63	1.56	1.35	2015
Kraków	1.24	2.50	0.68	0.66	1.02	1.51	1.09	1.24	
Kielce	2.50	0.49	0.63	1.70	1.11	0.82	2.79	1.44	2016
Kraków	1.86	1.02	1.02	3.59	0.86	0.41	4.85	1.95	
Kielce	5.20	1.10	0.41	1.26	1.35	3.22	3.38	2.27	2017
Kraków	4.09	1.25	0.53	0.85	1.14	4.25	2.68	2.11	

Bacterial cell count estimates

In spring, in autumn 2016 and spring 2017, the estimated bacterial cell count was 0.2 $\log_{10}$ CFU higher in truffle soil samples (Tab. 3) than in control samples. The largest difference (0.4 $\log_{10}$ CFU) in bacterial cell count between the variants was recorded in autumn 2017. The average number of bacteria in soils with truffle, regardless of the evaluation date, was 6.6 $\log_{10}$ CFU, while in control soils, it was slightly lower at 6.3 $\log_{10}$ CFU.

Table 3. Total bacteria count (CFU) and total actinomycetes cell count per 1 g of soil (T – truffle soil, C – non-truffle soil). Different letters indicate significant differences between the averages

Bacterial	Variant	Spring 2016	Autumn 2016	Spring 2017	Autumn 2017
Bacterial cell count estimates	T	6.5 b	6.5 ab	6.5 b	6.7 a
	C	6.3 c	6.3 c	6.3 c	6.3 c
Actinomycetes	T	6.5 a	6.4 a	5.7 c	5.2 d
	C	5.9 bc	6.0 b	5.1 d	5.2 d

In spring 2016, the total number of bacteria in the truffle variant was significantly higher than in the control variant at plot W only. A similar pattern was also observed in autumn 2016 for plots G and W (Fig. 2). In spring 2017, on plots M and W, a higher bacterial cell count was recorded in the truffle variant than in the control variant; in autumn of the same year, the soil bacteria count increased on all three plots.

Extremely low ranges of K coefficient in 2015 and in 2016, indicating drought in soil, probably also affected the number of bacteria. In spring 2017, the number of bacteria in the T plots was lower than in autumn 2017, after rainfall. The low ranges of the K weather factor recorded at the Krakow station in August and September 2016 did not have a positive effect on bacterial growth in both variants T and C – the quantitative increase was only recorded on the surface GT.

Actinomycetes

The average actinomycetes estimated cell count, regardless of plot location, in spring and autumn 2016 and in spring 2017 was higher in truffle soil than in control soil. In autumn 2017, the evaluated cell count of actinomycetes was similar in all variants (Tab. 3). The greatest difference between truffle and control variants was recorded in spring 2016 (0.6 $\log_{10}$ CFU) and the smallest in autumn 2016 (0.4 $\log_{10}$ CFU). The total bacterial cell counts in the control variant and the truffle variant in autumn 2017 were similar.

Average actinomycetes count was calculated for each of the sample locations in each evaluation period (Fig. 4). In spring 2016, the number of actinomycetes

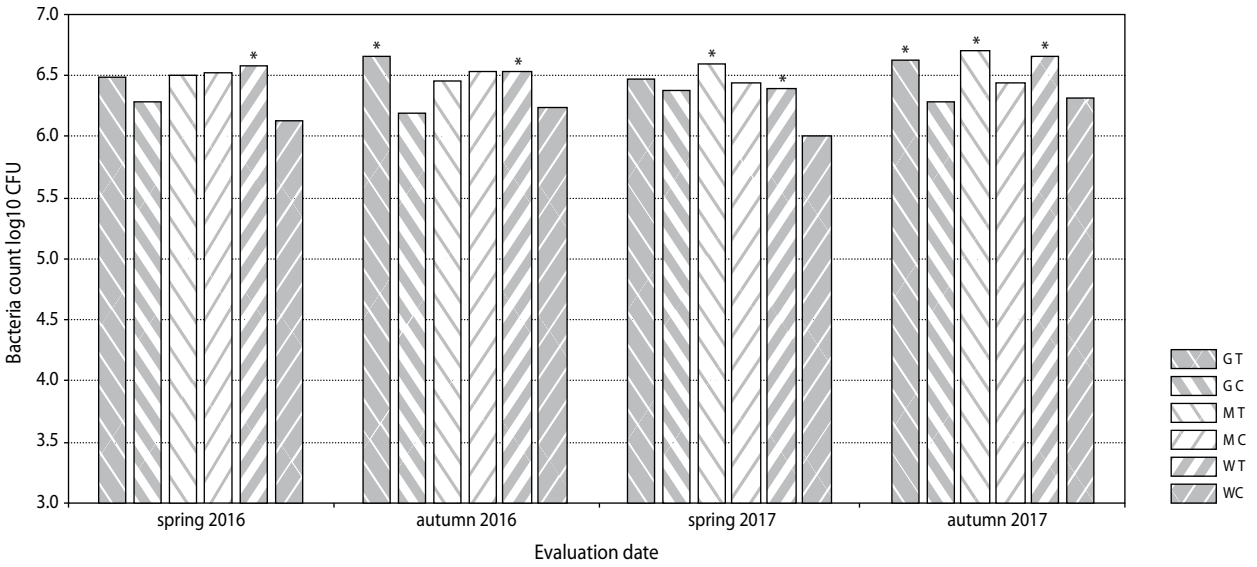

Figure 2. Bacterial cell count estimate ($\log_{10}$ x per 1 g of soil) in samples from each variant. The * star indicates significant differences in averages between the truffle soil variant (T) and the control soil (C)

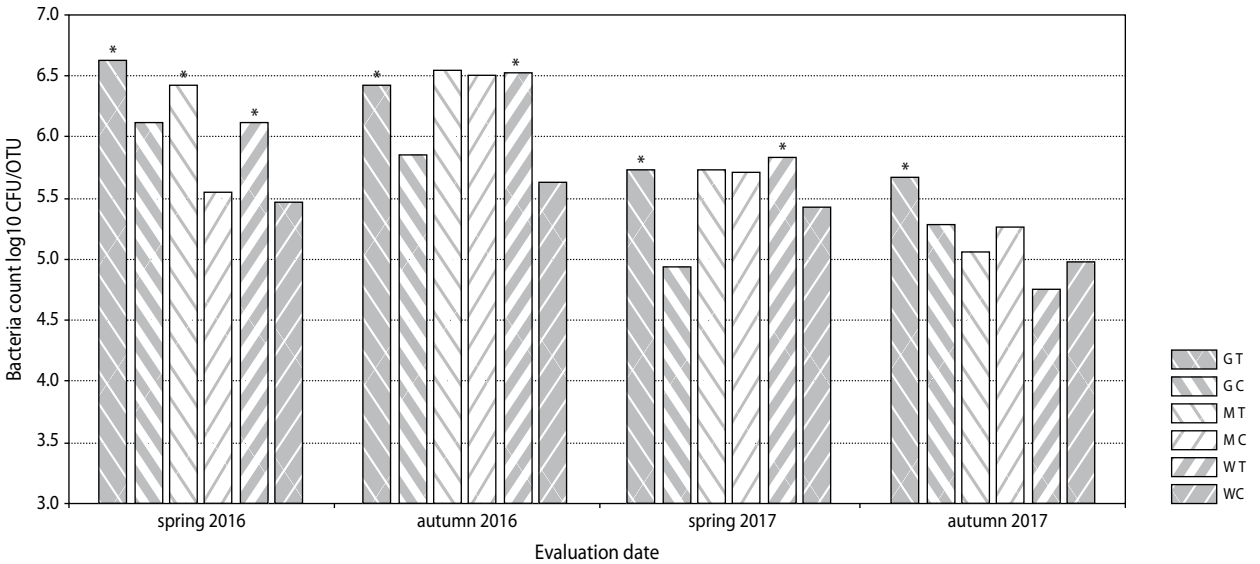

Figure 3. Average estimated cell counts of actinomycetes grouped by plot (G, M, W), the presence of truffle (T, C) and evaluation date (spring, autumn 2016 and 2017). The * symbol indicates significant differences between truffle (T) and control (C) soil samples

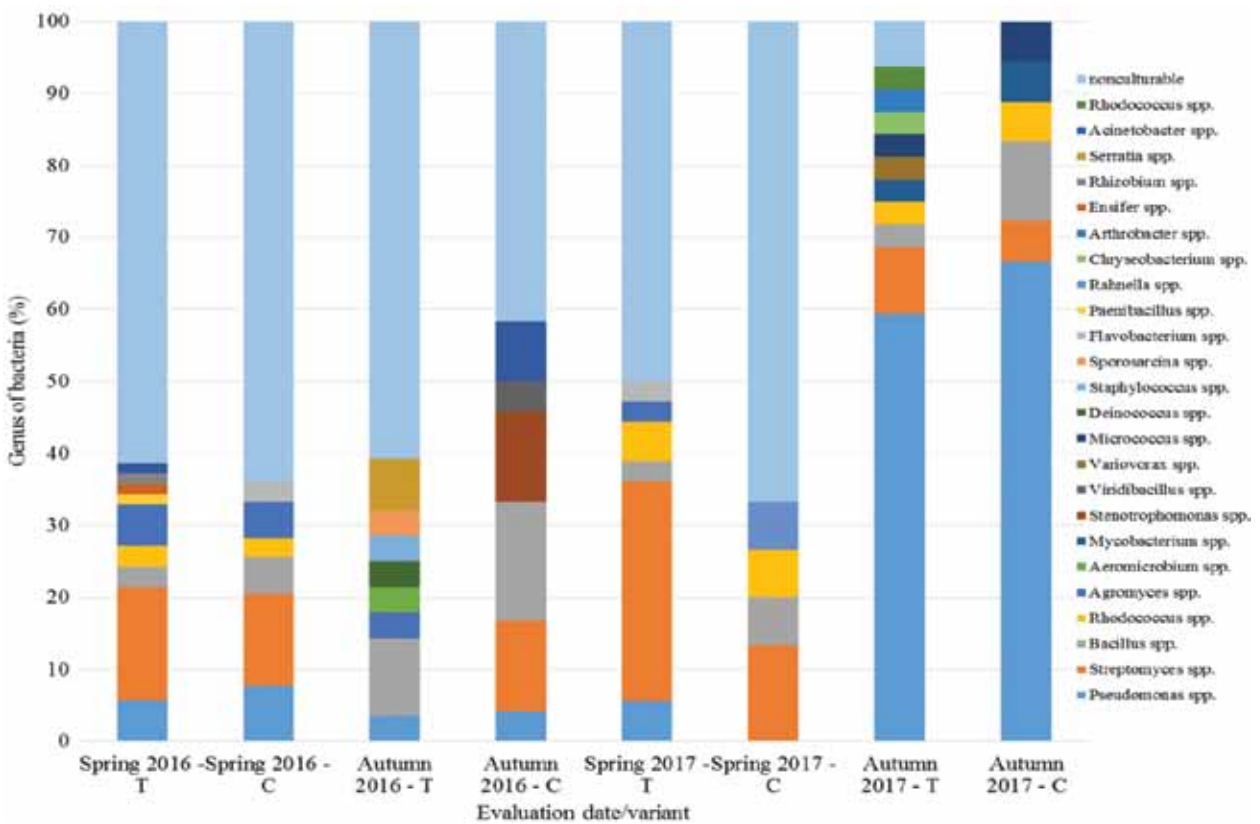

Figure 4. Percentage frequency (%) of bacterial biota in soils of the study sites T (sites with *T. aestivum* present) and C (control sites, no *T. aestivum*) and evaluation date

in truffle soils was greater than in control soils for all plots (Fig. 3), whereas in spring, this difference was significant only for plot G. In spring 2017, a significantly higher bacterial cell count was recorded in truffle soils from plots G and W; however, in autumn 2017, as in 2016, this was only true of plot G.

Due to the low estimated frequency of fluorescent bacteria in the soil, it was not possible to carry out a statistical analysis of their distribution, however, the confirmed presence of *Pseudomonas* bacteria is worth noting, as confirmed by qualitative analysis.

Qualitative PCR analyses

Out of the 347 clonal bacterial cultures isolated in 2016 and 2017 from all plots, 28.5% were nonculturable bacteria, while the remaining 71.5% were subject to molecular analyses. Seven classes of bacteria were identified through Sanger sequencing (Tab. 4).

The most numerous bacteria belonged to the genera *Pseudomonas* (class Gammaproteobacteria) (33%), *Streptomyces* (class Actinobacteria) (29%) and *Bacillus* (class Bacilli) (15%). Bacteria from the genera *Rhodococcus* and *Agromyces* each accounted for 6%, and various species from the genera *Aeromicrobium*, *Mycobacterium*, *Stenotrophomonas*, *Viridibacillus*, *Variovorax*, *Micrococcus*, *Deinococcus*, *Staphylococcus*, *Sporosarcina*, *Flavobacterium*, *Paenibacillus*, *Rahnella*, *Chryseobacterium*, *Arthrobacter*, *Ensifer*, *Rhizobium*, *Serratia* and *Acinetobacter* collectively accounted for 11% (Fig. 4). Figure 4 shows the generic names of several species found.

Table 4. Percentage frequency (%) of bacterial classes by year (2016, 2017), variant (T and C) and evaluation period (S for spring and A for autumn)

Class	Genus	2016				2017			
		T		C		T		C	
		S	A	S	A	S	A	S	A
Actinobacteria	*Aeromicrobium*	0.0	3.6	0.0	0.0	0.0	0.0	0.0	0.0
	Agromyces	5.7	3.6	5.1	0.0	2.8	0.0	0.0	0.0
	Mycobacterium	0.0	0.0	0.0	0.0	0.0	3.1	0.0	5.6
	Rhodococcus	2.9	0.0	2.6	0.0	5.6	3.1	6.7	5.6
	Streptomyces	15.7	0.0	12.8	12.5	30.6	9.4	13.3	5.6
Alphaproteobacteria	*Rhizobium*	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bacilli	*Bacillus*	2.9	10.7	5.1	16.7	2.8	6.7	3.1	11.1
	Paenibacillus	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sporosarcina	0.0	3.6	0.0	0.0	0.0	0.0	0.0	0.0
	Staphylococcus	0.0	3.6	0.0	0.0	0.0	0.0	0.0	0.0
	Viridibacillus	0.0	0.0	0.0	4.2	0.0	0.0	0.0	0.0
Betaproteobacteria	*Variovorax*	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0
Deinococci	*Deinococcus*	0.0	3.6	0.0	0.0	0.0	0.0	0.0	0.0
Flavobacteria	*Chryseobacterium*	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0
	Ensifer	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Flavobacterium	0.0	0.0	2.6	0.0	2.8	0.0	0.0	0.0
Gammaproteobacteria	*Acinetobacter*	1.4	0.0	0.0	8.3	0.0	0.0	0.0	0.0
	Pseudomonas	5.7	3.6	7.7	4.2	5.6	59.4	0.0	66.7
	Rahnella	0.0	0.0	0.0	0.0	0.0	0.0	6.7	0.0
	Serratia	0.0	7.1	0.0	0.0	0.0	0.0	0.0	0.0
	Stenotrophomonas	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0

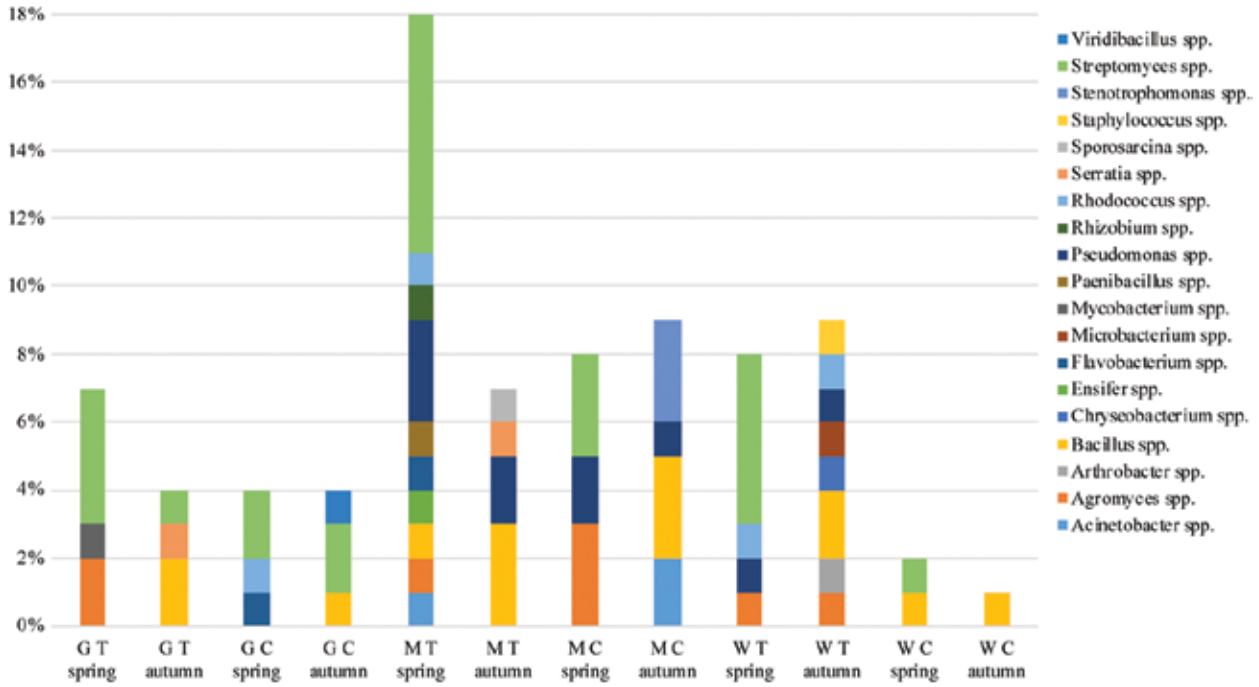

Figure 5. Percentage frequency (%) of bacterial species in 2016 sampled soils by examined variants

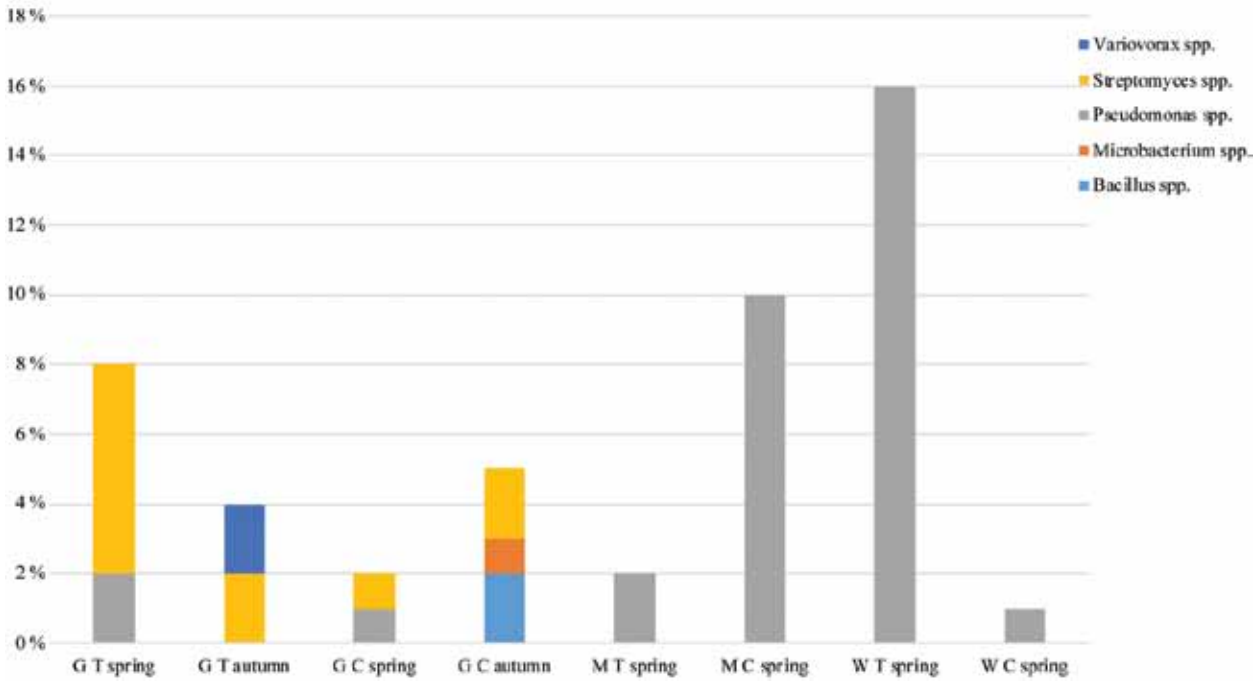

Figure 6. Percentage frequency (%) of bacterial species in 2017 sampled soils by examined variants

In 2016, *Streptomyces* were more numerous in truffle soils than in control soils. On plot M, in truffle soils, *Streptomyces* species share was higher in spring than in autumn, while in truffle soils from plot W, a greater share was recorded in autumn than in spring (Fig. 5). In 2017, *Streptomyces* were present both in the truffle and control variants regardless of the evaluation date, but they occurred only on plot G. On plots M and W, the soil was dominated by bacteria belonged to *Pseudomonas* genus. On plot M in spring, a greater share of *Pseudomonas* sp. was observed in the control variant, but on plot W, it was greater in the truffle variant (Fig. 6).

Discussion

Soil

According to Górska and Russel (2004), soil pH is an important regulator of microbial growth, because it affects the solubility of mineral substances and how they are assimilated by plants and other organisms living in the soil. In this study, the soil pH in truffle soil plots was between 5.9 and 7.1, and in non-truffle plots, it was between 3.5 and 7.0. From the analyses carried out by Gryndler et al. (2013) on soil samples collected from a hornbeam forest (*Carpinus betulus* L.), it follows that soil reaction has a significant effect on the growth and fructification of summer truffle. For stands dominated by hornbeam, similar results were obtained in this study. Similar to truffles, various types or species of microbes, including bacteria, require an environment close to their optimal pH (Hilszczańska et al. 2019a, b). The estimated bacterial cell count was higher in the truffle variant than in the control variant, which predicts a beneficial effect of alkaline soil (pH) on the development of bacteria (Kołwzan et al. 2005; Błaszczyk 2010). As the best conditions for bacteria include a pH of 6.5–7.5, the conditions in the selected soils of the Nida Basin proved to be favourable to them. The rendzic soil reaction (pH) was so similar that it did not affect overall bacterial diversity between soils with summer truffle ascocarps and control soils.

Although the pH of the soil did not affect the number of bacteria, in the case of an overall bacterial assessment, the weather conditions in the autumn season in 2017 probably influenced the community increase. Sielianinov's hydrothermal coefficient was then above 2, which indicates a high water saturation of the soil after the dry summer months in 2015 and 2016. An inverse relationship was noted for actinomycetes. Soil moisture contributed to a smaller increase in the number of actinomycetes in autumn 2017. It was found that the soil pH in samples with summer truffle fructification was higher than in samples with no ascocarps of this fungus.

In the Nida Basin soils, the availability of nutrients (e.g., N, C, K) could influence the growth of bacteria, depending on whether the environmental conditions were favourable for the development of truffles or not. It was found that the concentration of N and C was almost twice higher in the samples with truffle ascocarps recorded than in control soils from plots G and W. A similar situation was observed with the potassium content (K cmol/kg^{-1}), which was recorded as over three times more abundant in soils on plots GT and WC than in soils on GC and WC. The study conducted by Mello et al. (2013) on the chemical parameters of soil (e.g., N, K) did not show any differences between the soils in which summer truffles fructified and control soils. The differences in the content of Ca^{2+} ions among the truffle soil plots (e.g., W and G) were close to significant. The results indicate that the presence of these ions in the soil had an influence on the growth of this fungus, compared to the control soils, which were not conducive to it. The content of Ca^{2+} ions expressed in ‰ in plantations, where the stand age was 60 years, was almost twice as high as in soils with truffles, compared to soils without truffles (Mello et al. 2013).

The study by Mello et al. (2013) on the fructification of *Tuber melanosporum* in stands of pubescent oak (*Quercus pubescens* Willd.) investigated by DGGE and microarray, did not differentiate the soils with truffles and those without truffles in terms of soil pH and mineral composition. However, it showed a significant effect of the tree age, that is, ascocarps of *T. melanosporum* were more numerous in a younger stand (10–16 years) than in an older stand (60 years old).

Communities

This study employed two methods of analysis, classical cell culture for determining the cell count of selected groups of bacteria and actinomycetes, and a qualitative analysis to determine the diversity of the soil bacteriome. This approach showed all the differences

between the taxa of culturable and non-viable bacteria colonizing the forest soil, in which summer truffle ascocarps were found, and in soil without ascocarps of *T. aestivum*.

It is estimated that only 0.1–1% of microbes inhabiting soil biotas can grow on traditional media, which follows from classical quantitative analysis. Classical microbiological methods allow identification of only 10% of the composition of the bacterial population, as most soil bacteria are nonculturable or at least very difficult to culture (Sait et al. 2002). From among the bacteria isolated in 2016–2017 from soil samples from the Nida Basin, the nonculturable bacteria accounted for as much as 28.5% of all evaluation variants. This proves that the microbes of this biocenosis are very little known. Our explorations allowed us to estimate the bacteria count in the areas where the ascocarps of summer truffles are present and to identify the genus of the bacteria, although only 71.5% of bacteria were culturable. Traditional breeding methods have so far allowed characterizing bacteria by examining individual strains under laboratory conditions. The classical analyses provide little information on the environmental biology of soil bacteria. This lack of appropriate molecular methods is usually the biggest problem in the examination of bacterial communities in soil (Kozdrój 2013).

In our study conducted in the Nida Basin, the number of fluorescent bacteria isolated on artificial selective media and present in the examined soils was so small in comparison to the total number of bacteria and actinomycetes that a statistical analysis was impossible. The study conducted by Mamount and Olivier (1992) showed a positive effect of *Pseudomonas* bacteria on the synthesis of *T. melanosporum*. The mycorrhizal fungal infection alleviated after 6 months and was very mild after 12 months. According to the mentioned authors, isolates of *Pseudomonas* bacteria were more efficient than others and favoured symbiosis with truffle, protecting truffle from its competitors and inhibiting the growth of phytopathogens. In the future, a measurable benefit from this knowledge may be the production of bacterial inoculations that would stimulate the yielding of truffles and other crops.

A comparison of the total number of bacteria and actinomycetes showed significant differences between variants in a given season. In both truffle and control soils, the genera in bacterial communities also differed from season to season. The use of molecular methods, namely DNA sequencing, showed that, in soil samples from the sites in question, the dominant populations of bacteria were that belonging to genera: *Pseudomonas* (class Gammaproteobacteria), *Streptomyces* (class Actinobacteria) and *Bacillus* (class Bacilli – type Firmicutes). This confirms the report by Błaszczyk (2010), who categorizes the bacteria detected in the soil to seven main phylogenic groups: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Planctomycetes, Verrucomicurobia and Acidobacteria, thanks to the use of molecular techniques.

Deveau et al. (2016), by comparing the counts of bacteria classes associated with the soil environment of truffles, obtained results similar to those of our study, but the shares of individual bacteria were slightly different. In this study, bacteria of the genus *Pseudomonas* (class Gammaproteobacteria) accounted for 33% of soil bacteria, and in the quoted study, only 2%. A higher percentage of bacteria examined in the Nida Basin was also recorded for the classes Actinobacteria (genus *Streptomyces,* 29%) and Bacilli (genus *Bacillus*, 15%). In the studies conducted by Deveau et al. (2016), the count of Actinobacteria class was more than 15% and the Bacilli class was less than 5%.

The study conducted by Mello et al. (2013) in the pubescent oak stands (*Quercus pubescens* Willd.), on the diversity of bacteria associated with ascocarps of *T. melanosporum* and employing the DGGE technique, allowed identifying four dominant classes and genera of bacteria: Actinobacteria, Alphaproteobacteria, Betaproteobacteria and *Bacillus*. *Pseudomonas* bacteria belonging to the class Gammaproteobacteria did not occur in large numbers.

Deveau et al. (2016) carried out a study on a truffle plantation in Rollainville, France, on bacterial diversity in an environment in which another truffle species (*T. melanosporum*) was present and the dominant species in the stand was common hazel (Corylus avellana L.). Although the authors did not analyse the physicochemical composition of the soil, one can assume that these soils differed from the soil in the Nida Basin. These differences are likely to influence the qualitative composition of bacteria, in particular, in soil samples of the study sites T (sites with *T. aestivum* present) and C (control sites, no *T. aestivum*).

Using the Sanger DNA sequencing method, bacteria from the genera *Rhodococcus* (6%) and *Chryseobacterium* were only recorded in autumn 2017 in truffle soils. Bacteria from the genera *Aeromicrobium* and *Deinococcus* were found in truffle soils in autumn 2016. In autumn 2017, no bacteria from the genus *Pseudomonas* were found in any samples. In the paper by Gryndler et al. (2013), bacteria from the genus *Rhodococcus* were also identified, and the 454 sequencing allowed the identification of the strain *Rhodococcus maanshanensis* FR750959. Frey-Klett et al. (2007) and Gryndler et al. (2013) report that bacteria from the genera *Rhodococcus*, *Streptomyces,* and *Arthrobacter* promote the growth of mycelium and may influence the formation of truffle ectomycorrhiza.

Despite these numerous findings, there is still little research-based information confirming the direct relationship between the presence and activity of various bacteria and their effect on the growth of truffles. It is believed that bacteria from the genera *Rhodococcus*, *Streptomyces,* and *Arthrobacter* are among the typical mycorrhizal helper bacteria (Maier et al. 2004; Frey-Klett 2007; Schrey et al. 2007; Lehr et al. 2008).

The milestones obtained from these studies can be summarized as follows:

- the distribution of truffle ascocarps is significantly influenced by the content of calcium ions in the soil, which was on average 42.6 cmol/kg^{-1}, and almost twice higher than in the control variant (without truffles ascocarps) – 24.06 cmol/kg^{-1};
- in truffle soils, the average bacterial load was similar – 6.6 log10 CFU and in control soils, 6.3 log10 CFU, and average frequency of actinomycetes was 6.0 log10 CFU in truffle soils and 5.6 log10 CFU in control soils;
- *Agromyces* bacteria share was predicted by both truffle presence in the soil and the period of observation: in 2016, in truffle soils – 4.6% on average and in control soils – 2.6%, and in 2017, in truffle soils – 0.9% on average, whereas in the control soils *Agromyces* were not detected;
- *Pseudomonas* spp. frequency was not found to be associated with the date or season; in 2016, the difference between relevant representation in truffle and control variants was 1.3%, and in 2017 it was 3.1%;
- traditional methods used so far in soil microbiology do not answer the questions posed.

Conclusions

The obtained results indicate an underestimation of the qualitative and quantitative composition of bacteria population 'accompanying' truffles in an assessment with traditional methods. Therefore, in order to clarify the ecological position of bacteria associated with *T. aestivum*, it seems necessary to use new techniques for isolation and identification. One such method involves understanding the metagenome of bacterial communities associated with the soil environment of truffles. Consequently, should to be an interesting premise for continuing research in the field of enzymatic activity of soil bacterial communities, as well as their participation in the formation of natural mycorrhizal systems with summer truffle, and could help to supervise the bacterial communities in truffle plantations in order to increase the yield of ascocarps.

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