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Metabolic activity of soil bacteria associated with summer truffle *Tuber aestivum*

Marta Siebyła[✉]

Department of Scientific Information and Promotion, Forest Research Institute, Sękocin Stary,
Braci Leśnej 3, 05-090 Raszyn, Poland

ABSTRACT

This study investigates the biological activity of bacteria in soil samples collected from sites with summer truffle (*Tuber aestivum*) (T) ascomata and control sites (C) without *T. aestivum* at three selected sites (G, M, W) in southern Poland. The number of fragments of the 16S rRNA gene in isolated DNA samples was determined by real-time PCR (qPCR) as a measure of bacterial abundance. Soil metabolic activity was assessed using the BIOLOG system. It was hypothesised that the soil samples studied would differ in terms of the enzymatic and molecular activity of the bacteria inhabiting them. The results obtained for the samples of plots G and W showed differences in bacterial abundance between the two variants. Nevertheless, in plots G and M, the metabolic activity of the microbial communities, as determined by the AWCD index after 100 h of incubation, was higher by ~0.04 and ~0.1 AWCD units, respectively, in the control variant than in the soil samples with *T. aestivum*. This implies that studies of bacterial communities in soil should consider both copy gene numbers and community activity as measured by the AWCD index.

The results of this study deepen the knowledge about the bacterial communities and their metabolic activity in soils with *T. aestivum* and their potential influence on the formation of *T. aestivum* ascomata.

Introduction

Soil microbial communities are highly variable in terms of abundance and activity, and the degree of this variation, irrespective of the genetically determined characteristics of the organism concerned, depends on several factors. Among these, physical and chemical soil properties, soil moisture, pH, temperature, nutrient availability and the influence of the plants present in the area stand out (Curtis *et al*., 2002; Robe *et al*., 2003; Pociejowska *et al*., 2014; Pent *et al*., 2017). According to Van Elsas *et al*. (2006) and Roesch *et al*. (2007), soil microorganisms have the highest level of prokaryotic diversity. The biodiversity of microorganisms and their metabolic activity are therefore important factors influencing soil productivity and stability (Hooper *et al*., 2005). Delmont *et al*. (2011) estimated that 1 gram of soil contains up to 10 billion microorganisms rep-

resenting thousands of species. The abundance of bacteria in forest soils and forest litter is about $4.8×10^9$ and $7.5-9.5×10^8$ cfu, respectively (Torsvik *et al.*, 2002; Krivtsov *et al.*, 2005; Ipsilantis *et al.*, 2007; Błaszczyk 2010; Siebyła *et al.*, 2017). The most abundant microbial community in the soil can be found in the plant root zone. The number of taxonomic groups of bacteria in the rhizosphere in 1 gram of soil can be as high as $1.2×10^9$ cfu, whereas in non-rhizosphere soil it is $5.0×10^7$ cfu (Benton *et al.*, 2002; Eggleton *et al.*, 2005; Flyn *et al.*, 2009; Culman *et al.*, 2010; Flohre *et al.*, 2011).

Soil bacteria are responsible for the interaction of the abiotic (temperature, humidity) part of the soil with plants by producing and releasing the nutrients such as nitrogen uptake, production the different hydrolytic enzymes, IAA, etc. needed for plant growth and development. In turn, microbial growth is facilitated by the secretion of organic substances and chemical compounds from plant roots, including carbohydrates, amino acids, organic acids, vitamins, phenolic compounds, enzymes and sterols (Galus-Barchan *et al.*, 2014; Siebyła *et al.*, 2017). Tilak *et al.* (2005), Egamberdiyeva (2007), Huang *et al.* (2014) showed that secreted organic compounds can be chemoattractants for specific groups of microorganisms in the immediate vicinity of roots. These include substances secreted by the roots of *Arabidopsis thaliana* (L.) Heynh., which attract *Bacillus subtilis* (Ehrenberg) Cohn. bacteria and improve the condition of the diseased plant. Zhang *et al.* (2014) showed that citric acid secreted by roots stimulated the *Bacillus amyloliquefaciens* (Fukumoto) Priest *et al.* strain SQR9 to form a biofilm around the roots, whereas fumaric acid played a similar role in the *B. subtilis* strain N11.

Błaszczyk (2010) reports that Actinobacteria commonly found in the soil environment, but also in fresh and salt waters, composts and manure, decompose animal remains and biological materials such as cellulose, chitinase, or xylanase via enzymes such as cellulase, chitin and lignin. According to the author, these bacteria also produce antibacterial, antiviral, antifungal, anticancer and anthelmintic compounds.

The soil zone directly surrounding the plant roots creates favourable conditions for the development of various organisms, including belowground fungi of the genus *Tuber* (truffle). Nine species of truffles have been confirmed in Poland, of which four species are valued for their culinary value: summer truffle *Tuber aestivum* Vittad., large spore truffle *T. macrosporum* Vittad., hollow truffle *T. mesentericum* Vittad. and white truffle *T. borchii* Vittad. (Hilszczańska, 2016). Although the species most frequently recorded in Poland is *T. aestivum*, studies on the microbiome associated with this species are scarce. The metabolic activity of the bacteria accompanying the truffle in the places, both in the soil and in the ascomata, is also not fully known. Perlińska--Lenart *et al.* (2020) identified bacteria and fungi associated with the ascomata of *T. aestivum* and examined the 16S rDNA V3-V4 region after sequencing PCR products using an Illumina MiSeq. According to Jankiewicz *et al.* (2015), under laboratory conditions, *T. aestivum* secretes extracellular enzyme proteins with lactase, protease, lipase, xylanase and cellulase activities.

Proteolytic enzymes and lipases are probably involved in obtaining energy components for the growth of the truffle mycelium, whereas xylanases, cellulases and lactases are likely to be involved in the establishment of mycorrhizal symbioses (Jankiewicz *et al.*, 2015). Hence, the enzymatic and molecular activity of soil from confirmed summer truffle sites and of soil without truffles (control) was analyzed. Real-time PCR (qPCR) analyses for 16S rRNA gene fragments were applied to determine the number of target fragments of this gene in DNA samples isolated from the soil, which would serve as a measure of bacterial abundance in the studied samples. It was also assumed that the analysis performed simultaneously using the metabolic BIOLOG™

method, designed for environmental studies (Gryta *et al.*, 2014), would facilitate the assessment of the ability of bacteria to assimilate and use different carbon substrates (carbohydrates, carboxylic and keto acids, amino acids, amines and amides, polymers). The result of increased microbial metabolic activity is, among others, an increased dehydrogenase activity (Wolińska *et al.*, 2012; Gryta *et al.*, 2013), which can easily be assessed in the laboratory through the reduction of colourless tetrazolium violet to coloured formazan (Gryta *et al.*, 2013).

The results of Suz *et al.* (2008) and Gryndler *et al.* (2013) on rDNA gene copy number of *T. aestivum* in soil using qPCR to compare *T. melanosporum* Vittad. and *T. aestivum* species show that a quantitative approach to molecular studies using qPCR is important for practical reasons. Summer truffle ascomata obtain carbon from the host tree through mycorrhiza, but there are no studies on the saprotrophic capacity of this species (Barry *et al.*, 1994). Here, tested the hypothesis that differences in bacterial abundance in locations where summer truffle ascomata are found, and the degree of the activity of metabolic processes in this soil (based on the degradation of carbon sources) would allow for the identification of bacterial groups that facilitate the growth of truffles in a given area.

It was hypothesised that the number of bacteria in the sites where the summer truffle ascomata were detected will be different depending on the area (stand community) and that the activity level of metabolic processes in the control variant will be different (lower) than in the variant where the summer truffle cultures are growing.

We hope that the results of the analyses of the soils of the studied plots will reveal the different living conditions of truffles and the formation of their ascocarps, as well as the accompanying bacterial communities. This will be due to differences in the chemical composition of soils, species composition of plants of a particular biotope, or specific meteorological conditions (Siebyła *et al.*, 2020, 2021). We also hope that the results will provide a scientific reference for monitoring *T. aestivum* and indicate soil conditions favorable for its introduction into plantations.

Materials and methods

SAMPLING. The study was carried out in three research plots [G, M and W, with *T. aestivum* (T) ascomata and without *T. aestivum* (control; C)], located in the Nida Basin (342.27 Nida Basin Mesoregion) (Solon *et al.* 2018) in fresh or upland mixed forests growing on rendzina-type soil (Hilszczańska, 2016; Siebyła *et al.*, 2020). A detailed description of the soil (pH, elemental composition, humidity and temperature) can be found in other paper Siebyła *et al.* (2020). In the study, the areas were marked as GT and GC, MT and MC as well as WT and WC. In plots M and W, the predominant forest habitat type was upland forest, and in plot G – fresh forest. In plot W, the dominant tree species were *Quercus petraea* (Matt.) Liebl. (85 years*), Acer pseudoplatanus* L. and *Carpinus betulus* L.; whereas in plot M, *Quercus robur* L. (140 years), *Tilia cordata* Mill., *C. betulus* and *Fagus sylvatica* L. dominated. In plot G, the species *C. betulus* (100 years) and *Q. robur* were predominant. The bedrock in plot G (gypsum rock) also differed from those in plots M and W (calcareous marl) (Hilszczańska *et al.*, 2014).

For soil metabolic analyses and the determination of soil bacterial abundance at the above locations (G, M and W), soil samples were collected in spring 2017 from a depth of 15 cm (18 samples in total). Three subsamples from each plot were combined to obtain a composite sample. For the T subplots, these subsamples were G1-G3, M1-M3, and W1-W3. For the C subplots, the subsamples were G4-6, M4-M6, and W4-6. Prior to the analyses, the soil samples were stored at –20°C to avoid uncontrolled multiplication of the bacterial groups present.

Molecular analysis of soil samples – qPCR of 16S rRNA gene fragments. The DNA isolation was performed using 0.5 g of each of the 18 soil samples (three replicates per sample), according to the instructions for the Genomic Mini AX Soil Spin isolation kit (A&A Biotechnology). The isolated DNA, 150 µl in volume, was stored at –20℃ and then used for real-time polymerase DreamTaq DNA polymerase (Fermentas, Thermo Scientific) chain reaction PCR.

The specific primers used were pF (5'-ACG AGC TGA CGA CAG CCA TG-5') and pE (5'-AAA CTC AAA GGA ATT GAC GG-3'), and the reaction was performed using a LightCycler 96 (Roche Diagnostic GmbH, Mannheim) (Loeffler *et al.*, 2000; Hein *et al.*, 2001). The composition of the reaction mixture for DNA amplification by real-time PCR was as follows: LightCycler® 480 SYBRGreen MasterMix 2x conc. (Roche Diagnostic GmbH) – 5 µl, primer pF (10.0 pmol, Sigma Aldrich) – 0.25 µl, primer pE (10.0 pmol, Sigma Aldrich) – 0.25 µl, sterile water (LightCycler® 480 SYBRGreen I Master H_2O PCR Grade) – 3.5 µl, analysed DNA – 1 µl.

The real-time PCR reaction of the 16S rRNA gene fragment started with preincubation, *i.e.*, preheating of the reaction mixture at 94℃ for 10 minutes. Subsequently, each of the 30 cycles was carried out according to the following procedure: denaturation at 95℃ for 10 seconds, annealing at 57℃ for 20 seconds, elongation at 72℃ for 30 seconds. After each cycle, the amount of amplification product was determined, using the fluorescence signal of the SYBRGreen dye. To validate the PCR methodology, amplification was also performed on a negative control sample not containing the template DNA (standard).

Analysis of soil samples using the BIOLOG™ system. The functional diversity of soil bacteria was assessed using the BIOLOG method on EcoPlates™. These plates, used in environmental studies, contain 31 different carbon sources in triplicate. The level of consumption of individual carbon sources by bacteria is expressed by the change in the colour activity of suspended solids in the wells (Insam, 1997; Loeffler *et al.*, 2000; Hein *et al.*, 2001; Gryta *et al.*, 2013).

To perform the analysis, soil equivalent to 10 g of dry matter was weighed from each sample and suspended in 90 ml of sterile saline solution. The suspensions were shaken for 60 min at 120 rpm, and from the obtained soil suspensions, a 10-2 dilution was prepared in saline, of which 120 µl were applied to the wells of the EcoPlates™. The plates were incubated at 24℃ for 5 days. Immediately after the suspension was added and at intervals of 12 hours, the colour of the suspension in each well was measured at 590 nm, using a spectrophotometer.

The measured absorbance of the blank well was adjusted, and the absorbance value from the first measurement was subtracted. Based on the adjusted results for each plate, the average well colour development (AWCD) values were calculated to determine the metabolic activity value of the microbial communities, using the following equation: AWCD=Σ substrate absorbance/31, where 31 is the number of carbon sources used (Gryta *et al.*, 2014).

The AWCD curves were plotted against the incubation time for each of the soils tested and the use rate of each of the 31 substrates was calculated as the area under the curve (AUC):

$$AUC = A_0 + A_1 / 2x (t_0 + t_1) + A_1 + A_2 / 2x (t_1 - t_2) + \ldots\ldots + A_{n-1} + A_n / 2x (t_n - t_{n-1}),$$

where:

AUC – area under the curve,
Tn – reading time, *h*,
A_n – absorbance value at the time t_n,
and the average area under the curve (AAUC) (AAUC=ΣAUC/31).

Based on the AUC and AAUC values, the following indices were calculated:

a) The Shannon biodiversity index (diversity, $H'=-\Sigma pi(lnpi)$), which describes the degree of functional diversity of a community, where pi is the ratio of the AUC value of a given substrate to the sum of the AUC values of all the substrates in the replicate,

b) metabolic richness (Rs), i.e., the number of substrates degraded,

c) evenness ($\Gamma=H'/logRs$), where H' – Shannon biodiversity index, Rs – number of substrates degraded in a given repetition (richness) of occurrence of metabolic group bacteria in the assembly. The evenness index takes values from 0 to 1, where 1 represents even degradation of all substrates by the bacteria.

SUBSTRATE DEGRADATION PROFILES ON ECOPLATES. Substrate degradation profiles were expressed as area under the curve (AUC), determined after 100 hours of incubation. Substrates were placed according to the manufacturer's instructions.

STATISTICAL ANALYSES. Two-factor analysis of variance of the tested plots (G, M, W) and variants (T, C) was applied to assess the average 16S rRNA gene copy number in each system. To determine differences between profiles of a given plot and variant, MANOVA was performed after normality of decomposition was established using the Shapiro-Wilk test. To reveal differences between mean 16S rRNA gene copy number in soils where *T. aestivum* ascomata were found, post-hoc analysis was also performed using the NIR test. Correlation analysis was used to determine differences between plots along the PC1 and PC2 axes. Based on the substrate degradation profiles, principal components analysis (PCA) was performed after 100 hours of incubation. Data were analysed using Statistica version 10 (Statsoft, 2011).

Results

MOLECULAR ANALYSIS OF SOIL SAMPLES – qPCR OF 16S rRNA GENE FRAGMENTS. *Post-hoc* NIR analysis showed significant differences between the mean 16S rRNA gene copy number in the soil where *T. aestivum* ascomata were observed and in the control soil for the two plots M and W. The 16S rRNA gene copy number in plots G and W for soils with *T. aestivum* ascomata was higher than that in the control.

There was a significant difference between the means for the plot (M, G, W) × variant (T, C) interaction, as well as for the plot × variant interaction in the soil with truffle and in the control soil for plots M and W (Fig. 1). The highest number of gene copies (10.2 log10) was recorded in the soil of the WT plot and the lowest in the MT plot (9.7 log10). For the control variant, the highest number of gene copies (10.0 log10) was recorded in plots M and G, whilst the number of gene copies in plot W was 0.1 log10 less (9.9 log10). In plot G, no significant differences were found in terms of the 16S rRNA gene copy number between the control soil and the soil with *T. aestivum*.

ANALYSIS OF SOIL SAMPLES USING THE BIOLOG™ SYSTEM
Metabolic activity. The values of the AWCD index, which determines the metabolic activity of the community, increased with increasing incubation time in all three plots and in each of the three replicates, albeit in a different manner.

The metabolic activity of the bacterial communities, as determined by the AWCD index values, increased linearly until 24 h of incubation for plot G. After this time, samples G3 and G4 showed the highest metabolic activity levels (>1.00 AWCD units), with no significant differences between these two samples (84 h). Sample G5 was characterised by intermediate values (approximately 0.80 AWCD units – 96 h), whereas the remaining samples, including soils from

the two *T. aestivum* plots (G1, G2) and from G6, showed considerably lower values, although a similar metabolic activity (96 h) (Fig. 2).

In the soils representing plot M, the metabolic activity of the bacteria increased continuously, with an almost linear increase from 36 to 72 h of incubation (Fig. 3). The AWCD values for the control soil (samples M4, M6) were similar (about 0.5 AWCD units) and more than twice as low as those for the soil with *T. aestivum* (samples M2-M3).
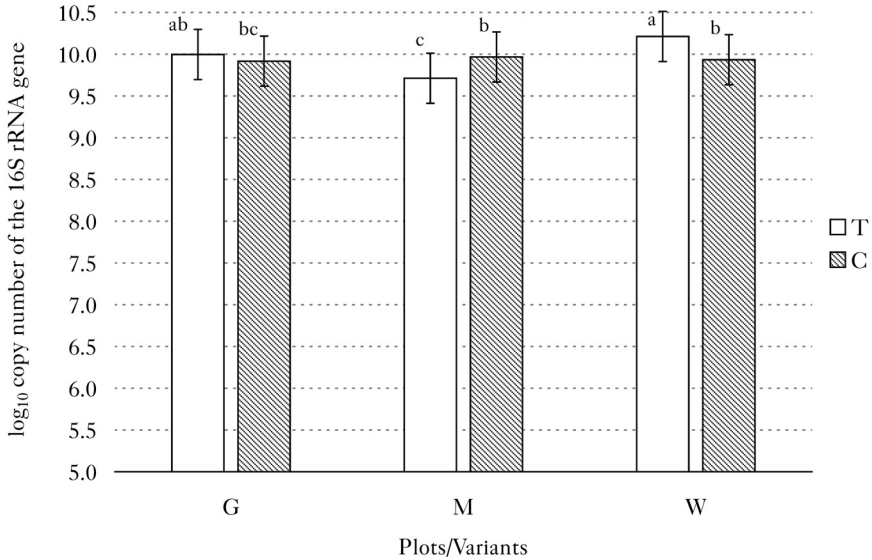

Fig. 1.

16S rRNA gene copy number in G, M and W plots for soil with truffle (T) and control (C). Labels with different letters indicate significant differences between means
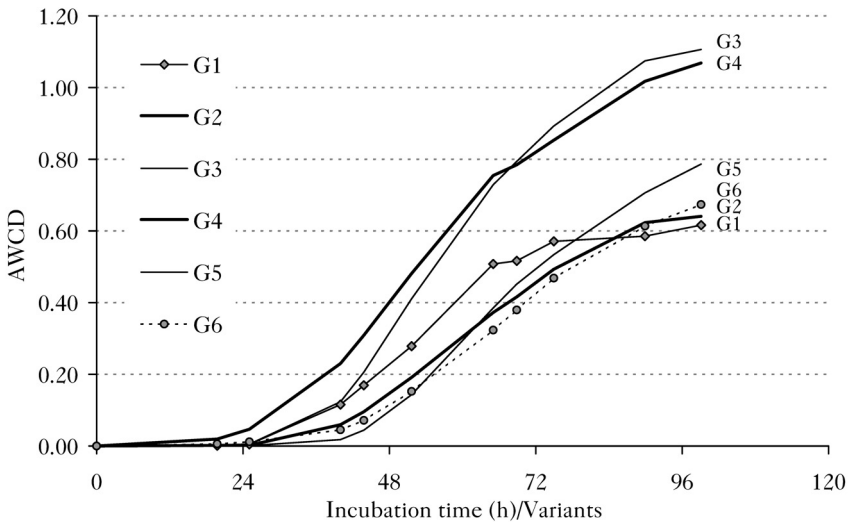

Fig. 2.

Metabolic activity of microbial communities using the AWCD index after 100 h of incubation for soil samples from plot G. Samples G1-G3 contained *T. aestivum* (T), samples G4-G6 are the control samples (C)

In plot W, soil metabolic activity (>96 h) was generally lower than in plots M and G (metabolic activity AWCD index below 1.0), with lowest values for W5. In contrast, the soils of control samples W4 and W6 and of W3 showed the highest AWCD index values (around 0.8) (Fig. 4).

The value of the AWCD index determining the metabolic activity at the end of the experimental period (after 100 hours) significantly differed among the plots ($p=0.016$). Plot W showed the lowest value (~0.60) and plot G the highest one (~0.80). Regardless of the plot, the bacterial
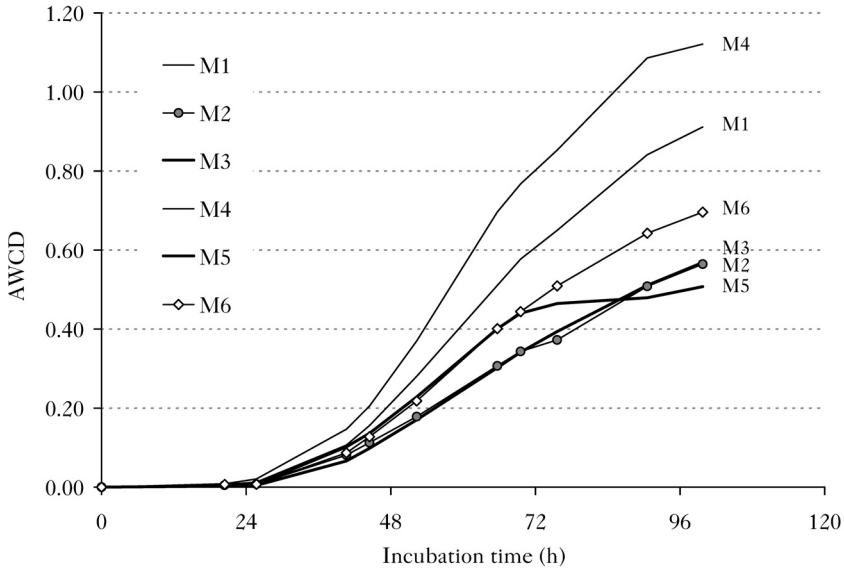

Fig. 3.

Metabolic activity of microbial communities using the AWCD index after 100 hours of incubation for soil samples from plot M. Samples M1-M3 contained *T. aestivum* (T), samples M4-M6 are the control samples (C)
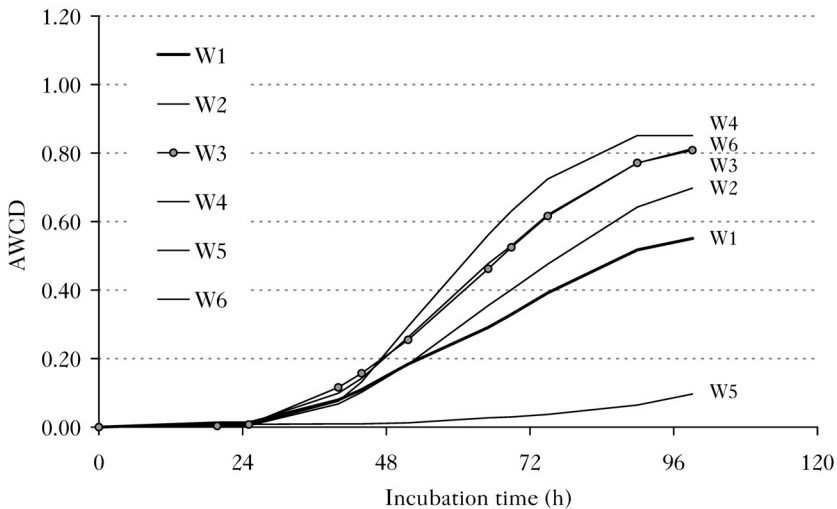

Fig. 4.

Metabolic activity of microbial communities using the AWCD index after 100 hours of incubation for soil samples from plot W. Samples W1-W3 contained *T. aestivum* (T), samples W4-W6 are the control samples (C)

activity expressed by the AWCD index value for the control soil was always slightly higher than that for soil with *T. aestivum* (Table 1).

There were only significant differences between the means for the Shannon index (*H'*) (*p*=0.03); the value for the WC control plot (1.23 units) was significantly lower than those for the WT plot and the other variants (Table 1).

The degree of bacterial activity in the soil optimal for *T. aestivum* development (variant T) was highest in samples from plot G (0.78 units) and lowest for plot M (0.66 units). The results presented (Fig. 5) confirm that in plot G, the degree of bacterial activity was most conducive to *T. aestivum* development, whereas in plot W, a medium (~0.7 units) degree of community activity was found (Fig. 5). The microbial activity of the control soils at all locations was higher than that of the soil with *T. aestivum*, with the highest (<0.8 units) activity recorded in the WC plot (Fig. 5). Bacterial activity was highest in plot G, regardless of the variant assessed.

Diversity of the bacterial communities. The degree of functional diversity (*H'*) of the bacterial community in plot M was similar for both variants (MT and MC), that the presence of *T. aestivum* had no impact on the diversity of the bacterial community. In plot G, the Shannon index value

Table 1.

Values of biodiversity indices and the AWCD index for the three plots M, G and W, including variants T (soil with *T. aestivum ascomata*) and C (control soil without *T. aestivum*)

Samples	H'	Rs	I'	AWCD
	p=0.03	*p*=0.16	*p*=0.07	*p*=0.24
GT	1.30 ±0.07a	24.44 ±4.07a	0.94 ±0.02a	0.79 ±0.25a
GC	1.31 ±0.08a	26.22 ±3.53a	0.92 ±0.03a	0.84 ±0.18a
MT	1.30 ±0.06a	26.56 ±3.61a	0.92 ±0.02a	0.68 ±0.19a
MC	1.30 ±0.07a	25.89 ±3.95a	0.93 ±0.02a	0.77 ±0.29a
WT	1.32 ±0.04a	26.78 ±2.17a	0.93 ±0.02a	0.69 ±0.12a
WC	1.23 ±0.15b	24.11 ±5.86a	0.90 ±0.04a	0.59 ±0.38a

Different letters within columns indicate significant differences between the means
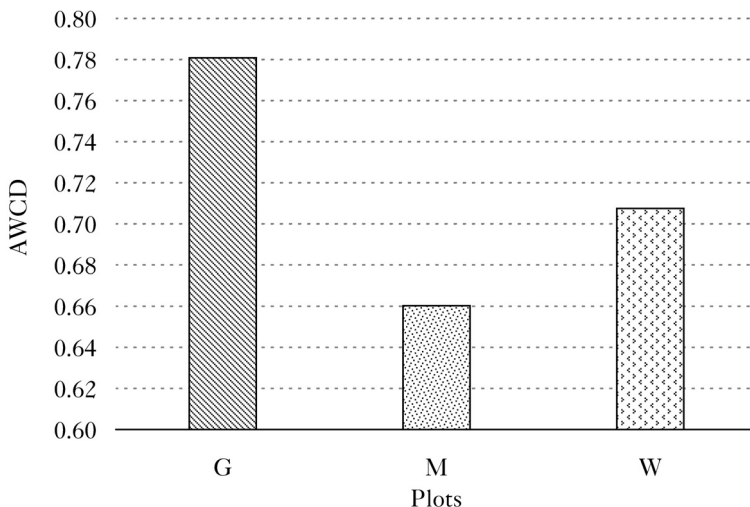

Fig. 5.

Average metabolic activity of microbial communities based on the AWCD index after 100 hours of incubation for soil samples from plots G, M and W, including the variant T (soil with *T. aestivum* ascomata)

in the control variant was even 0.013 units higher than that in the soil containing *T. aestivum*. However, there was an inverse relationship for plot W, where the degree of functional diversity in soil with *T. aestivum* was 0.093 units higher than that in the control variant (Table 1).

The metabolic richness of the bacterial communities (Rs index) in plots M and W was higher in the variants with *T. aestivum* compared with the control variants (Table 1). However, in plot M, the difference between T and C was only 0.667 units, whereas in plot W, this difference was 2.667 units.

The values of the evenness index (I') for the occurrence of metabolic groups in the bacterial complex showed that the substrates were degraded most significantly in the GT samples (0.937 units), with the lowest degradation rates in WC soil (0.897 units). A value of 1 would imply an even degradation of all substrates. Although the statistical analysis showed no difference in the plot × variant interaction, plot variation was marginally significant at $p=0.07$.

Regarding the values of AWCD, Rs and I', no differences were found between variants (T and C). However, an influence of the plot location factor was identified, without taking the variant into account ($p=0.016$). In terms of the AWCD index values, the mean metabolic activity of the bacteria colonising the soil in plot G (0.82 units) was significantly higher than in plot W (0.64 units), which was confirmed by the NIR test.

SUBSTRATE DEGRADATION PROFILES ON ECOPLATES AND STATISTICAL ANALYSES
Principal components analysis of the substrate degradation profiles indicated that the first component (PC1), differentiating the profiles by the degree of substrate degradation, explained 71.53% of the variation between profiles, whereas the second component (PC2), indicating the carbon source used, explained 28.47% (Fig. 6). Cases located on the negative side of PC1 (GT, MC) indicated a higher degree of substrate degradation compared to those located on the positive side of PC1. The soils of MT and WT, with the presence of *T. aestivum*, were characterised by a high
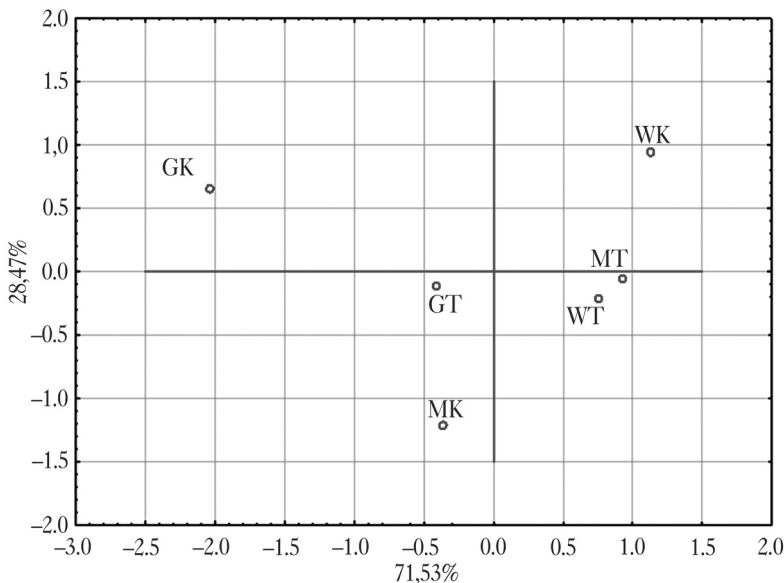

Fig. 6.
Principal components analysis for physiological profiles of soils by plots (G, M, W) in samples soil with *T. aestivum* ascomata (variant T) and in the control soil without *T. aestivum* (variant C)

similarity of physiological profiles. In contrast, the GC control soil was negatively correlated with the WC control sample, indicating that the control samples differed from each other, similar to the soils with and without *T. aestivum*.

Based on the MANOVA results, plot location was the only significant factor differentiating the physiological profiles of the bacterial communities studied (p=0.0273). The mean substrate

Table 2.

Microbial activity expressed by the degree of plate colour, taking into account the plot and variant, at 590 nm, and correlations of the studied carbon sources with the principal components (PC1 and PC2) in the studied soils according to BIOLOG^{TM} analysis, taking into account the plots (M, G, W) and variants (T – soil with *T. aestivum ascomata*, C – control soil without *T. aestivum*)

Groups	Substrates	Microbiological activity						Principal components	
		G T	G C	M T	M C	W T	W C	PC 1	PC 2
Amino acids	L-Arginine	40.94	34.57	38.57	44.86	33.29	34.56	–0.91	*0.31*
	L-Asparagine	71.21	75.23	61.80	63.64	57.02	61.86	–0.84	0.05
	L-Phenylalanine	1.73	4.18	2.76	4.37	2.26	2.05	–0.72	0.08
	L-Serine	54.72	59.77	44.88	56.79	45.12	46.96	–0.91	–0.08
	L-Threonine	1.13	1.79	1.80	1.63	2.88	1.89	–0.11	–0.14
	Glycyl-L-glutamic Acid	1.72	6.18	2.09	4.62	6.58	3.66	–0.64	0.01
Amines/ amides	Phenylethylamine	27.31	16.98	22.39	26.06	19.33	14.27	–0.90	0.18
	Putrescine	36.85	29.85	34.75	40.57	37.77	30.09	–0.86	*0.44*
Carbo- xylic and keto acids	D-Galactonic Acid γ-Lactone	54.72	47.61	48.28	58.79	54.01	35.36	–0.84	0.21
	D-Galacturonic Acid	62.12	54.12	65.21	72.62	69.41	46.86	–0.59	<u>0.50</u>
	2-Hydroxy Benzoic Acid	0.24	1.49	1.86	3.64	0.41	0.00	–0.61	–0.03
	4-Hydroxy Benzoic Acid	54.87	23.48	35.01	46.94	33.41	28.53	–0.79	0.29
	γ-Hydroxybutyric Acid	27.96	22.66	43.66	30.89	31.95	25.48	–0.74	*0.45*
	D-Glucosaminic Acid	20.75	30.13	17.00	15.73	19.00	19.38	–0.83	–0.25
	Itaconic Acid	28.61	36.18	21.07	14.84	20.65	25.62	–0.70	–0.28
	α-Ketobutyric Acid	0.00	0.58	0.03	0.44	0.00	0.61	–0.42	0.08
	D-Malic Acid	26.85	30.40	36.09	51.90	38.01	29.55	–0.71	*0.38*
Polymers	Tween 40	31.98	39.33	34.06	39.60	32.15	28.68	–0.87	0.01
	Tween 80	43.09	46.36	47.38	51.04	52.41	39.11	–0.73	*0.37*
	α-Cyclodextrin	5.12	3.61	3.63	10.72	7.53	3.79	–0.79	0.17
	Glycogen	8.39	7.02	5.58	9.88	10.13	8.71	–0.79	0.05
Carbo- hydrates	ß-Methyl-D-Glucoside	47.40	64.78	21.28	47.90	37.47	45.52	–0.64	–0.42
	Pyruvic Acid Methyl Ester	47.50	47.86	46.61	48.62	43.33	32.12	–0.92	0.05
	D-Xylose	10.07	13.69	5.27	7.43	2.62	3.06	–0.75	–0.27
	i-Erythritol	7.11	12.21	7.34	3.71	4.22	0.68	–0.33	**–0.58**
	D-Mannitol	71.53	83.98	54.04	64.97	49.14	43.93	–0.81	–0.35
	N-Acetyl-D-Glucosamine	60.60	79.88	38.52	60.65	50.51	52.59	–0.75	–0.42
	D-Cellobiose	49.45	27.20	8.70	38.15	5.84	24.74	–0.68	–0.10
	Glucose-1-Phosphate	12.71	29.57	5.12	18.00	5.29	3.32	–0.58	**–0.70**
	α-D-Lactose	25.32	32.13	5.43	8.98	4.00	3.41	–0.69	–0.39
	D,L-α-Glycerol Phosphate	8.43	12.66	7.55	10.90	8.72	6.63	–0.61	–0.38

Underline indicates a positive correlation of degraded D-galacturonic acid with the plot (r=0.5),
bold indicates a high (r>0.5) negative correlation for glucose-1-phosphate and i-erythritol,
italics indicates an average (0.3<r<0.5) correlation between the degraded substances hydroxybutyric acid, putrescine, malic acid, Tween 80 and L-arginine

degradation profiles from a given plot differed, as demonstrated by their location relative to the PC2 axis ($p<0.05$). Post-hoc analysis of the NIR test showed that the mean profiles obtained from plot G took negative values on the PC2 axis and differed from those obtained for plots W and M, indicating that in terms of soil physiological profiles, plot G differed from plots M and W.

The variation between plots could be explained by the analysis of the PC2 component (explaining 28.47% of the variation in the correlation of substrates with the plot × variant). There was a high ($r>0.5$) negative correlation of glucose-1-phosphate with i-erythritol, expressed by the position relative to the PC2 axis (Table 2). This means that in plot G, these substrates were distributed to a greater extent than in the other plots. In contrast, a high positive correlation was observed for D-galacturonic acid ($r=0.5$) and an average correlation ($0.3<r<0.5$) for hydroxybutyric acid, putrescine, malic acid, Tween 80 and L-arginine, as indicated by their location relative to the PC2 axis. On average, these substrates were distributed to a greater extent in plots W and M than in plot G, with higher values in the control soil compared to the soil with *T. aestivum* (Table 2).

Discussion

Based on the results of real-time PCR, the bacterial 16S rRNA gene copy number was higher in soil with *T. aestivum* (plots G and W) than in the control soils. This confirms the results obtained by Mello *et al.* (2013) using PhyloChip analysis, who found a higher number of OTUs in soil samples from *T. melanosporum* ascomata sites compared to control sites. Such results indicate a similar trend in the relationship between truffle and control soils, regardless of the *Tuber* species present, leading us to infer that the bacterial communities present have a positive effect on truffle occurrence. The higher abundance of bacteria in the truffle variant also indicates their higher – the variability indicator, which has also been reported by Gryndler *et al.* (2013). In a similar study, Siebyła *et al.* (2020), using culturing techniques to identify bacteria, also showed differences in bacterial abundance between soils with *Tuber* (6.6 log 10 cfu) and a control soil (6.3 log 10 cfu). In this study, real-time PCR analysis showed that bacterial abundance in the truffle variant ranged from 9.6 log 10 gene copy numbers (plot M) to 10.2 log 10 (plot W).

In this study, components analysis for physiological profiles obtained from plot G shows their differentiation in relation to profiles obtained from plots M and W. Samples GT, GC and MC were characterised by a higher degree of substrate degradation compared to WT, WC and MT. Analysis of the physiological profiles showed a high correlation of soil locations with substrates. A strong negative correlation was observed for carbon sources such as glucose-1-phosphate and i-erythritol, indicating that these substrates were distributed to a greater extent in plot G, which was located in mixed forest, compared to plots W and M, located in upland forest. In contrast, other substrates such as hydroxybutyric acid, putrescine, malic acid, Tween 80 and L-arginine were distributed to a greater extent in plots W and M than in plot G. As reported by Colombo *et al.* (2014) organic acids, including malic acid, are involved in the release of phosphorus compounds from forms inaccessible to bacteria.

Some bacteria stimulate the formation of root tuber ectomycorrhizae, which may result in an increased flow of organic carbon into the soil, stimulating the growth of mycelium in truffle soil (Dominguez *et al.*, 2012). Metabolites that are a source of carbon for the microorganisms surrounding the roots are released by the plants in the rhizosphere zone (Bais *et al.*, 2006; Hartmann *et al.*, 2009; Hinsinger *et al.*, 2009). The quantity and quality of plant secretions depend on the species and the plant age, along with the physicochemical conditions of the soil, among other factors. Most likely, these differences it could be result from differences in the composition of tree

species in these 3 locations. The metabolic activity of roots affects the release of organic substances such as amino acids, organic acids, phenolic compounds, polysaccharides and proteins as well as oxygen or water. Borymski (2019) and Huang *et al.* (2014) showed that some compounds released by plants can act as attractants for microorganisms in the vicinity of roots, *e.g.*, *Pseudomonas fluorescens* Migula WCS365, in the case of amino acids that are secreted by the tomato root tip. This chemotaxis allows the bacteria to colonise the roots of this plant (Weert *et al.*, 2007). Another example is *B. amyloliquefaciens* (ex Fukumoto 1943) Priest *et al.* strain SQR9, isolated from the rhizosphere of seed cucumber, which showed the presence of a metabolite, citric acid, that stimulates bacterial movement towards the roots of the host plant. In addition to the chemotaxis effect, citric acid also stimulates *B. amyloliquefaciens* strain SQR9 to form a biofilm (Zhang *et al.*, 2014). Rudrappa *et al.* (2008) report that L-malic acid secreted by *A. thaliana* roots improved the condition of diseased plants by stimulating *B. subtilis*.

Although the soil physicochemical conditions, including mean soil pH (6.9 and 7.5 pH units, respectively), were similar in plots M, G and W (Hilszczańska *et al.*, 2019), the metabolic activity of the communities differed in all three plots. The soils supporting *T. aestivum* growth differed in terms of the mean metabolic activity of the microbial communities as measured by the AWCD index value. The different findings for plot G may be due to differences in forest type, bedrock, pH and meteorological conditions (Hilszczańska *et al.*, 2014). In plot G, the bedrock was gypsum rock, the dominant forest type was fresh forest, and the soil pH (7.03) was lowest compared to plots M and W (Siebyła *et al.*, 2020). In contrast, plots M and W were dominated by upland forest, the bedrock was marl and limestone, and the soil pH was 7.17 and 7.37, respectively.

Soil from plots with *T. aestivum* differed in terms of mean metabolic intensity. The highest standard deviation of this index was recorded in plot G with 0.78, followed by plot M with 0.57 and plot W with 0.37. The differences in mean metabolic activity may have been influenced the influence of different atmospheric precipitation on these surfaces.

Gomez *et al.* (2006), in an analysis of physiological profiles in clay and loam soils, reported differences in the functional diversity of soil microorganisms between the experimental variant and the control. Similar results were obtained in the present study and recorded type of soils was rendzic. In addition, the values of average well-colour development (AWCD) were higher in the soil of the control variant than in the soil with *T. aestivum*, which is in agreement with the findings of Gomez *et al.* (2006). From the study of Gomez *et al.* (2006), the difference between the control variant and the experimental plot was 0.371. In both studies, the value of the Shannon index differed between the variants.

Differences in the functional diversity of bacterial communities were noted only for plot W. Further, the metabolic richness of bacterial communities did not differ between plots and variants (with average r values of 24-26), indicating that the metabolites of the bacterial communities had no impact on the development of *T. aestivum* ascomata. However, Gomez *et al.* (2006) described some variability in this trait between experimental and control variants. Gryta *et al.* (2014), assessing the physiological profiles of sewage sludge after 100 hours of incubation, showed the usefulness of the AWCD index for differentiating between control and heavy metal-contaminated soil samples. The evenness index in metal-contaminated sludge was higher compared to that in control samples, whereas an inverse relationship was observed for metabolic richness (Gryta *et al.*, 2014). The evenness index in the present study and in the study by Gryta *et al.* (2014) was close to 1, indicating that high bacterial activity does not favour truffle growth.

Although the metabolic activity and evenness of substrate degradation by bacteria were highest in plot G, substrate degradation was lowest, most likely because of the lower adaptive capacity of soil microorganisms from the control soil compared to the soil with *T. aestivum*. Siebyła *et al*. (2020) report that the chemical composition of the analysed soil samples in the Nida Basin was significantly affected by the elements N, C and K as well as the Ca^{2+} levels. In this study, the proportion of these substances in plots G and W differed between the soil with and without *T. aestivum*.

According to Niemi *et al*. (1996), the number of carbon substrates used by soil microorganisms increases with increasing organic C levels in the soil. However, the results obtained by Siebyła *et al*. (2020) indicate the opposite effect; in plots G and W, where the percentage of carbon was almost twice as high in soil with *T. aestivum* compared to the control soil, the metabolic activity AWCD was lower in *T. aestivum* soil than in the control soil. A similar situation was observed for the percentage of nitrogen. Niemi *et al*. (1996) suggest that the activation of processes related to carbon and nitrogen degradation occurs by stimulating the development and activity of soil microorganisms, which is associated with an increase in the catabolic potential of the soil.

The findings from this study contribute to the improved understanding of bacterial communities and their metabolic activity with regard to *T. aestivum* and their potential influence on the formation of ascomata of this species. In addition, they contribute to a better understanding of their productivity under the controlled conditions of truffle plantations. Further research on the microbiome associated with truffles can facilitate the development of improved methods to quantify the presence of *T. aestivum mycelium* in soil and deepens the knowledge of the factors that support the proliferation and development of this species.

Conclusions

There was an overall higher bacterial abundance in soil with *T. aestivum* ascomata (T variants) compared to control soil (C). Greater degree of functional diversity of a community was observed, particularly in plots G and W, in the presence of *T. aestivum*. This leads me to infer that a higher bacterial degree of functional diversity of a community is conducive to the growth of *T. aestivum*. However, the bacterial species degree of functional diversity of a community as determined by the Shannon index, differed only between the sites in plot W, with a higher value in WT than in WC. The metabolic activity of the microbial community for plots G, M was higher in soil without the presence of *T. aestivum*, indicating that high microbial activity limits truffle growth. The degree of substrate (carbon compounds) degradation by bacteria was highest in plot G, irrespective of the presence of *T. aestivum*. In plot G, there were no differences between T and C soil in terms of 16S rRNA gene copy number, whereas in plot M, there were differences between T and C soil in terms of 16S rRNA gene copy number, but no differences in the metabolic activity of the microbial communities and the proportion of individual substrates in the physiological profile of the bacterial community.

Conflicts of interest

The author declare the absence of potential conflicts of interest.

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STRESZCZENIE

Aktywność metaboliczna bakterii glebowych towarzyszących trufli letniej *Tuber aestivum*

Praca dotyczy analizy aktywności biologicznej zbiorowisk bakterii w próbach gleb pobranych ze stanowisk występowania owocników trufli letniej *Tuber aestivum* (T) oraz stanowisk kontrolnych (C) w trzech wybranych lokalizacjach (oznaczonych jako G, M, W) w Polsce południowej. Miarą liczebności bakterii w glebie badanych stanowisk była liczba docelowych fragmentów genu 16S rRNA określonych w wyizolowanych próbkach DNA z wykorzystaniem metody PCR w czasie rzeczywistym (RT PCR). Ocenę aktywności metabolicznej gleby wykonano metodą BIOLOG. Przyjęto hipotezę, że badane próby gleb będą różnić się pod względem aktywności enzymatycznej i molekularnej zasiedlających je bakterii.

Ocena liczebności bakterii dla prób z lokalizacji G i W wykazała istotne różnice pomiędzy obydwoma wariantami (T i C) – liczba kopii genu 16S rRNA na wymienionych powierzchniach w glebie z truflą była wyższa niż w wariancie kontrolnym (ryc. 1). W lokalizacji W różnica liczebności bakterii pomiędzy wariantami wynosiła 0,3 log10 liczby kopii genu 16S rRNA, a na powierzchni G 0,1 log10 liczby kopii genu 16S rRNA. W przypadku lokalizacji M odnotowano natomiast odmienną zależność – większą liczbę kopii genu odnotowano w wariancie kontrolnym w porównaniu z glebą z truflą, a różnica wynosiła 0,3 log10 liczby kopii genu 16S rRNA. Stopień zróżnicowania funkcjonalnego zbiorowisk bakterii określony na podstawie wskaźnika Shannona różnicował badane powierzchnie, jednakże tylko w lokalizacji W, przy czym różnorodność gatunkowa zbiorowisk bakterii w wariancie gleby truflowej (WT) była wyższa w porównaniu z wariantem kontrolnym (WC) (tab. 1).

Aktywność metaboliczna zbiorowisk i mikroorganizmów, wyznaczona na podstawie wartości wskaźnika AWCD (Average Well Colour Development – średnia wartość absorbancji badanego związku mierzona przy długości fali 590 nm po 100 godzinach inkubacji), w wariancie kontrolnym powierzchni G i M była wyższa odpowiednio o ~0,04 i ~0,1 jednostek AWCD niż w próbach gleby, w których odnotowano obecność owocników trufli letniej. Może to wskazywać na ograniczające rozwój owocników oddziaływanie zbiorowisk bakterii o wysokiej aktywności mikro-

biologicznej, wynikającej z odmiennego składu gatunkowego badanej gleby (tab. 1; ryc. 2, 3). Na powierzchni W aktywność metaboliczna gleby była ogólnie niższa niż na pozostałych powierzchniach M i G (poniżej 1,0 intensywności metabolicznej wskaźnika AWCD), co może być efektem specyficznego składu tych zbiorowisk bakterii (ryc. 4). Średnia intensywność metaboliczna zbiorowisk mikroorganizmów (AWCD) oceniana w badanych próbach gleby (powierzchnie G, M, W z uwzględnieniem wariantu T) była najwyższa (0,78 jednostek) w próbach z powierzchni G, a najniższa (0,66 jednostek) dla powierzchni M (ryc. 5). Uzyskane wyniki potwierdzają, że na powierzchni G stopień aktywności zbiorowisk mikroorganizmów najbardziej sprzyjał obecności trufli.

Wyniki analizy korelacji badanych źródeł węgla ze składowymi głównymi (PC1 i PC2) w badanych glebach według analizy BIOLOG™ z uwzględnieniem powierzchni (M, G, W) oraz wariantów (T – gleba truflowa, K – gleba kontrolna) zamieszczono w tabeli 2. Przeprowadzona analiza głównych składowych profili rozkładu substratów wskazała, że pierwsza składowa główna (PC1), różnicująca profile ze względu na stopień rozkładu substratów na płytce EcoPlate, wyjaśnia aż 71,53% zmienności między profilami, wskazując na różnice pomiędzy wariantem T i C w trzech lokalizacjach (G, M, W), a składowa druga (PC2), opisująca źródło wykorzystywanego węgla – 28,47%, oznacza zmienność korelacji substratów powierzchnia × wariant (ryc. 6).

Przeprowadzone badania zwiększają zakres poznawczy występowania zbiorowisk bakterii i ich aktywności metabolicznej względem trufli letniej. Dalsze badania nad biomem bakteryjnym towarzyszącym truflom powinny przyczynić się do doskonalenia metod ilościowego oznaczania obecności grzybni *T. aestivum* w glebie oraz poznania czynników wspomagających rozprzestrzenianie się i rozwój tego gatunku grzyba w glebie.