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Bacterial Endospores as an Additional Source of Archaeological Knowledge in the Analysis of a Burial Cemetery of the Tarnobrzeg Lusatian Culture in Dębina (SE Poland)

Abstract

Trybała-Zawiślak K., Potocki L., Czopek S., Ząbek T. 2023. Bacterial Endospores as an Additional Source of Archaeological Knowledge in the Analysis of a Burial Cemetery of the Tarnobrzeg Lusatian Culture in Dębina (SE Poland). *Analecta Archaeologica Ressoviensia* 18, 117–129

Archaeological studies need to use laboratory techniques, including analytical methods like Atomic Absorption Spectrometry, gas chromatography – mass spectrometry, and high-performance liquid chromatography, as well as genetic methods to resolve and verify scientific hypotheses. However, additional tools are still needed in the case of the cultural practices and traditions of ancient societies. Archaeological examinations of cultural practices have made significant progress in recent years, but additional tools are still needed to fully understand the complexity and diversity of these practices. In this work, we demonstrate how the genotyping of soil bacteria that can produce endospores is a potentially additional method for discovering past funeral rituals in various human populations who used food during their ceremonies. Endospores were isolated from soil samples taken from inside earthenware cup and pot-type vessels from a burial ground identified with the Tarnobrzeg Lusatian culture (SE Poland). The detected species of spore-forming bacteria strains were mostly environmental (originating from soil and / or water). However, the presence of some of the taxa i.e. (*Peanibacillus, Bacillus*) may provide a valuable source of archaeological information. We found that a combination of molecular and microbiological analysis can support archaeological studies of burial grounds and – in particular – individual graves, especially when they are characterized by a complete lack of bones.

Keywords: archaeology studies, microbiological analyses, endospores, Tarnobrzeg Lusatian culture

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1. Introduction

Archaeological studies of burial grounds provide an invaluable source of information about human history, capturing details of migrations, habits, religions, and various tragedies such as wars and epidemics (Cano *et al.* 2000; Zink *et al.* 2003; Fernández 2012; Stantis *et al.* 2015). Unfortunately, one of the major problems of burial ground studies is their poor quality or lack of sufficiently preserved bones. This problem has often been described in the case of burial graves belonging to the Lusatian culture (Czopek and

Trybała-Zawiślak 2014) therefore, some alternative methods are still helpful in archaeological studies. The use of biological methods, including microbiological techniques, in the analysis and interpretation of studied samples from archaeological sites has changed research strategies in archaeology (Acinas *et al*. 2004; Andersen *et al*. 2012; Margesin *et al*. 2017). Currently, analysis of soil microorganisms is used to follow the variation in usable areas of archaeological sites, in the reconstruction of diachronic soil layers, the population density of an area, and also in determining the diet of a given ancient society, etc. (Mitusov *et al*. 2009; Moodley *et al*. 2009; Grund *et al*. 2014).

Another approach relies on the presence of microorganism species as a complementary technique in the dating of archaeological samples taken from the soil (Grund *et al*. 2014). Of particular interest is the use of microbiological tests to reconstruct the health of ancient populations (Spyrou *et al*. 2019). The isolation of microbial DNA from well-preserved soft tissue remains of humans and animals can constitute direct evidence of the presence of infectious diseases, the spatial distribution of the disease, the mutual relationship between host and pathogen, and allow the study of the evolution of pathogens (Rollo *et al*. 2006; Devault *et al*. 2014). In our analysis, we paid special attention to endospore-forming bacteria. In addition to their survival function under adverse environmental conditions, endospores can also play an important role in the long-term survival of a species (Nilsson and Renberg 1990). Bacterial endospores are very resistant cell structures of Gram-positive bacteria of the phylum *Firmicutes* (Onyenwoke *et al*. 2004; Wunderlin *et al*. 2013; 2014). Bacterial endospores are used in particular in paleoecology to investigate environmental changes by analyzing river sediments or samples taken from lakes (Lomstein *et al*. 2012; Wunderlin *et al*. 2014). Therefore, it seems reasonable to use microbial taxa capable of producing endospores as a complement to traditional archaeology methods based on artefact detection in order to obtain more information on ritualistic behavior concerning not only the Tarnobrzeg Lusatian culture (SE Poland), but also in detecting the anthropogenic activities of other cultures. A working hypothesis was that bacterial endospores embedded in the porous structure of ceramic vessels permanently altered the microbial content of the vessel (vascular microbiome). The research hypothesis was verified using the genetic analysis of bacteria capable of producing spores as a method that supports the archaeological research of boneless cemeteries. For this purpose, a comparative analysis of microorganism communities isolated from samples taken from archaeological vessels and environmental sources were performed. A soil sample taken from the vicinity of the grave was used as a control. Furthermore, Klindworth's designed degenerate primers were used for the detection of the 16S ribosomal RNA gene to reduce errors in the detection of microbial diversity during PCR studies (Klindworth *et al*. 2013).

2. Materials and Methods

2.1. Site Description and Soil Sampling

The samples were collected from the burial ground discovered in Dębina 6 (SE Poland) belonging to the Tarnobrzeg Lusatian culture. The date of the burial ground in Dębina was described as early $13^{th}/12^{th}$ century BC on the basis of the ^{14}C analysis with a probability of 2 sigma after calibration in some of the wood balk that was also found in the grave cavity (data not presented). We chose one of the graves, no. 47 (Fig. 1), of a typically irregular form situated along a N-S axis and 2.4–0.45/0.8 m dimension. Unfortunately, no skeletal bones were preserved, but the following burial accessories were found in skeletal grave no. 47: an undefined brown pin, two earthenware s-shaped pot-type vessels, one earthen bowl, one earthen cup, and one small earthen vase. All the burial accessories were located in the south part of skeletal grave no. 47, probably near the head (Fig. 2). The organization of burial no. 47 was characterized by a similar ceramic typology to other burials, as previously described by Czopek and Trybała-Zawiślak (2014). To increase the probability of the isolation of bacterial species from ancient endospores, it was assumed that bacterial spores could have been absorbed into the clay matrix of vessels, and therefore soil samples were taken directly from the bottom of the cup and pot-type vessel. Soil samples were kept at 4°C and were used for research several days after sampling. In the vicinity of the tomb, soil samples were also collected from four sites (at a distance of 1 meter from the grave) and used as controls.

2.2. Isolation of pure bacterial cultures from bacterial endospores and associated environmental bacteria from soil

All analyses were performed under sterile conditions. A soil solution was prepared by dissolving 1 g of the soil sample in 90 mL of physiological saline supplemented with the emulsifying agent Tween 80

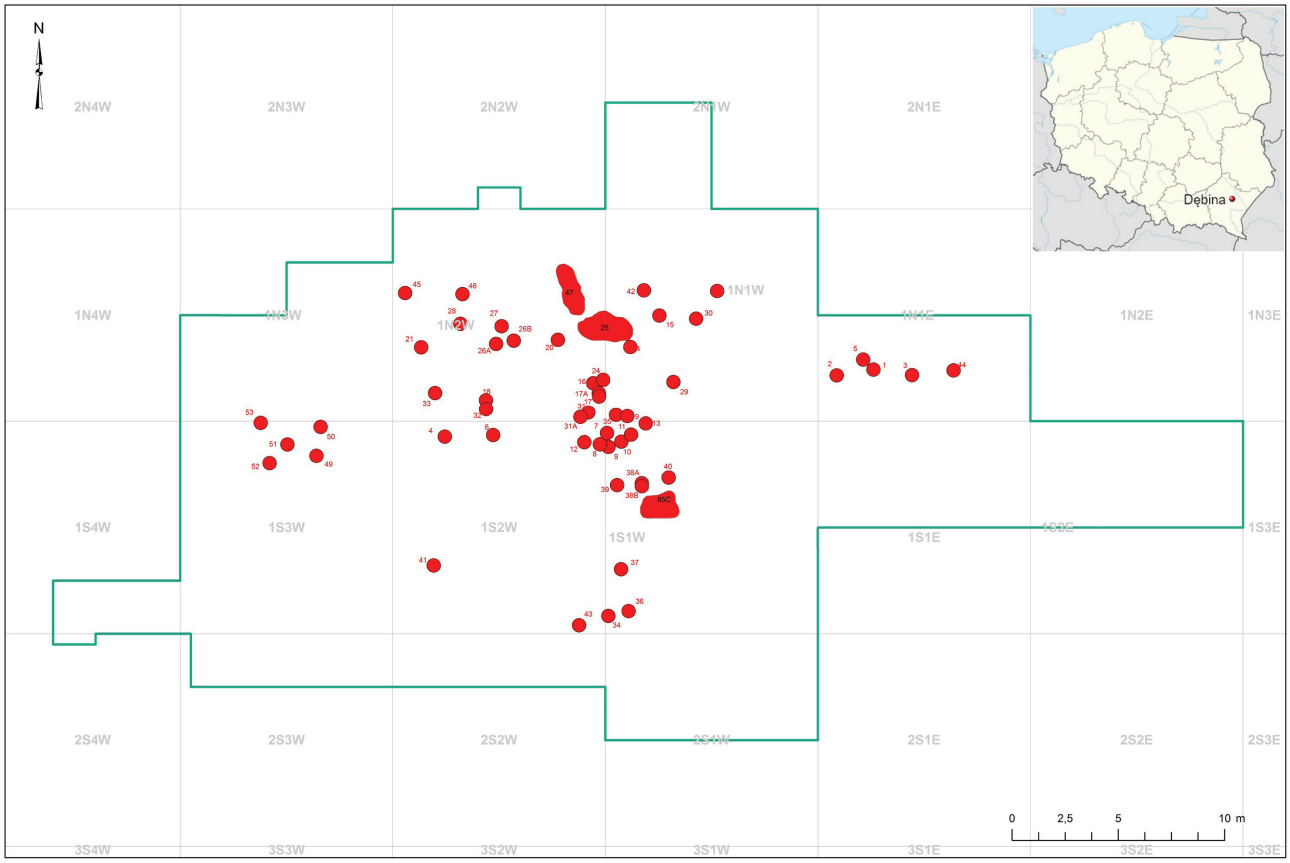

Fig. 1. Dębina, site 6, Poland – site localization and plan of cemetery with grave 47 (prepared by M. Jabłkowski).

(Sigma Aldrich, USA). The samples were shaken for 3 hours to elute the endospores and vegetative cells of soil microorganisms. In the next step, a serial dilution method was used to obtain single bacterial colonies. For this purpose, the obtained soil extract was then used to prepare fourteen-fold serial dilutions. Each 0.1 mL dilution was plated into Petri dishes with the nutrient agar in three repeats. To verify the presence of endospores in the soil solution, microbiological preparations were prepared and stained using the Schaeffer-Fulton method and observed under an Olympus BX43 light microscope at 1000x in oil immersion. In the next step, a soil solution was also used to prepare a spore extraction. Most bacteria cannot survive heating at 80°C for 10 minutes (pasteurization), unlike spores, which are thermally resistant and can survive for hours in boiling water. To do this, 1mL of the dilution (1:10, 1:100) was placed in tubs and incubated in the thermoblock for 15 minutes at 80°C to activate germination. To verify the presence of sporulation bacteria, 0.1 mL portions of each dilution were taken and spread on nutrient agar plates. Furthermore, inoculations on NA nutrient agar (meat extract – 10 g·L^{-1}, peptone – 10 g·L^{-1}, sodium chloride – 5 g·L^{-1}, agar – 20 g·L^{-1}, pH – 7.0) and Streptomyces GYM agar (glucose – 4.0 g·L^{-1}; yeast extract – 4.0 g·L^{-1}g; malt extract – 10.0 g·L^{-1}; CaCO3 – 2.0 g·L^{-1}; agar – 12.0 g·L^{-1}, pH – 7.2) were used as controls to differentiate vegetative bacterial species from the growth of bacterial species after the activation of the endospore germination process. Plates were incubated at 37°C for saprophytic and sporulation bacteria for 48 h and at 26°C for *Actinomycetes* for a period of 7 days. Colonies were chosen for the isolation DNA procedure on the basis of their morphological characteristics, therefore, picking similar colonies that developed on the plates at the same time was avoided. The isolated bacterial colonies were multiplied in nutrient broth at 37°C for 24 h. Furthermore, bacterial stocks of 40% v/v glycerol were taken from the prepared cultures and maintained at –80°C.

2.3. Preparation of genomic DNA from bacteria

Genomic DNA from each sample was isolated using a standard kit -GeneJet™ Genomic DNA purification Kit from Fermentas. The quality control of the isolated DNA was performed on 1% agarose gel.

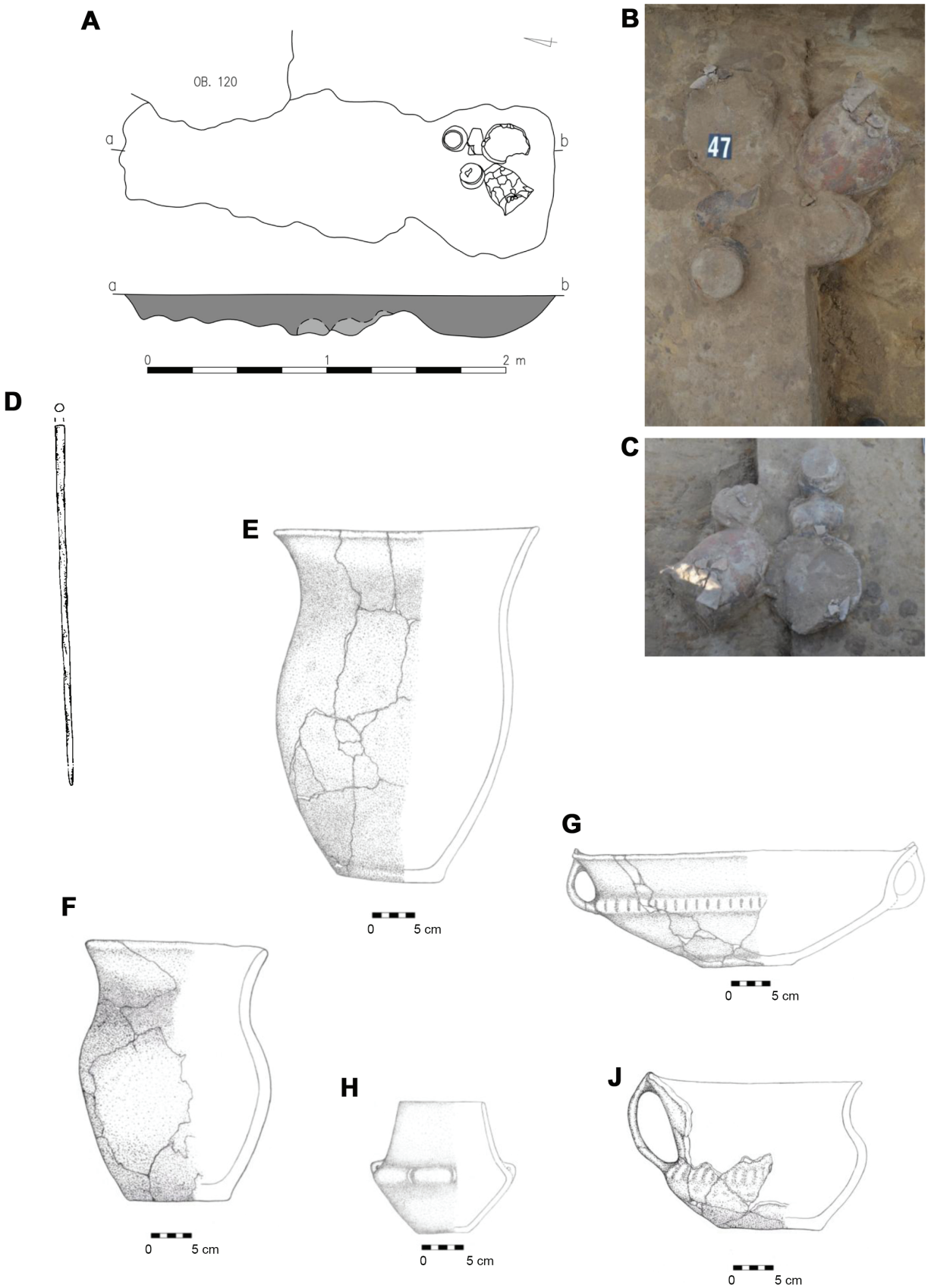

Fig. 2. Dębina, site 6, Poland – skeletal grave 47.
A – plan view and grave profile; B–C – vessels from the skeletal grave *in situ* (photo by K. Trybała-Zawiślak);
D–J – inventory of the skeletal grave (drawn by K. Trybała-Zawiślak).

2.4. Identification of isolated bacteria

To examine the microbial community specific to the archaeological samples, including activated bacterial species from endospores, we used sequence haplotyping in the locus encoding the 16S rRNA gene. For this purpose, we used specially designed degenerate primers, according to Klindworth *et al*. (2013). The most promising pair of bacterial primers proposed in population studies (SD-Bact-0341-bS-17(5'-CCTACGGGNGGCWGCAG-3')/SD-Bact-0785-aA-21(5'-GACTACHVGGGTATCTAATCC-3'), with an amplicon size of 464 bp, where the taxonomic distribution of 16S rDNA amplicons with 16S rDNA fragments was experimentally compared directly from the sequenced metagenomes. The PCR was carried out in 50 µL reaction volumes using 5 µL PCR buffer (10x) (Sigma Aldrich, USA), 1 µL $MgCl_2$ (100 mM) (Sigma Aldrich,USA), 1 µL dNTPs (10 mM each) (Sigma Aldrich, USA), 2 µL each of forward and reverse primers (10 µM) (Genomed, Poland), 0.5 µL Taq polymerase (5 U μL^{-1}) (Sigma Aldrich, USA), 2–3 µL DNA solution, and made up to the final volume with ddH_2O. The thermal cycling program was as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles consisting of denaturation (95°C for 40 s), annealing (2 min) and extension (72°C for 1 min) and a final extension step at 72°C for 7 min. The PCR products were purified using the GENEJET PCR purification kit (Thermo Fisher Scientific Inc., USA), and the cleaned PCR products were sequenced using Big Dye Terminator Cycle Sequencing Chemistry (Life Technologies, Poland) using PCR primers. The cleaned products of Sanger sequencing were subjected to capillary electrophoresis (Genomed, Poland). Sequencing reads were analyzed using the NCBI nucleotide sequence database search (BLASTN option) and interpreted using MEGA6.0 software. The phylogenetic tree was inferred using the maximum likelihood method based on the K2 model in MEGA software with 1000 bootstrap replicates. The image of a species-based phylogenetic tree was generated using the ETE toolkit (http://etetoolkit.org/treeview/) (Huerta-Cepas *et al*. 2016).

3. Results and Discussion

The main aim of this study was to determine whether bacterial spores of the *Firmicutes* genus can be used as "time-space capsules" to reveal past events, with potential applications in paleontology, paleoecology, and archaeology. We also focused on the following questions: which group of microorganisms grew in an environment rich in organic compounds and whether the archaeological vessels may have been filled with food and then deposited in the grave during ritual ceremonies. The last premise in particular is supported by the shapes of the archaeological vessels excavated from the grave no. 47 in Dębina. Additionally, control cultures on nutrient agar, Streptomyces GYM agar, and environmental bacteria from samples taken in the vicinity of the grave were used to identify spore-forming bacteria species that grew in an environment rich in organic compounds. Sequence analysis of 16S bacterial rDNA isolated from the bacterial soil collected from outside the earthenware vessels allowed us to identify the environmental background, which were excluded from the analysis. We also isolated species specific to archaeological samples with 99% sequence similarity to Genbank entries (Tab. 1; Fig. 3).The assignment of ambiguity for some species isolates is due to the high sequence similarity of the gene encoding 16S RNA. Our research also shows that the species composition of the bacteria isolated from the samples is fundamentally different. As a result, the comparative analysis showed that the most characteristic types of bacteria in the soil sample taken from the cup were: *Paenibacillus* (*Paenibacillus alginolyticus*, *Paenibacillus chondroitinus*, *Paenibacillus frigoriresistens*, *uncultured Paenibacillus sp.*) and *Psychrobacillus psychrodurans*. Similarly, the most abundant bacterial species in the soil sample taken from the pot were: *Acinetobacter radioresistens* and the genus *Bacillus* (*Bacillus marisflavi*, *Bacillus aquimaris*, *Bacillus vietnamensis*) (Fig. 3, 4).

The genus *Paenibacillus*, capable of producing endospores, may be particularly important in bioarchaeological studies by providing valuable information on the contents of cult vessels. It has been suggested that the *Paenibacillus* genus may cause fatal diseases in honeybees; while other species are opportunistic pathogens associated with human infections and others cause spoilage of pasteurized dairy products (Alippi *et al*. 2002; Sáez-Nieto *et al*. 2017). In addition, the genus *Paeanibacillus* is one of the bacterial genera regularly identified in soil microbiomes and enriched in close proximity to plants. It has been documented that throughout domestication, maize seeds have maintained a common set of bacteria with wild ancestors and each other, including *Paenibacillus* (Johnston-Monje and Raizada 2011).

A study of the maize seed microbiome also showed that a member of the *Paenibacillaceae* family originates from germinating maize seeds and not from the soil (Beirinckx *et al*. 2020). Interestingly, the pres-

Table 1. Presence of different types of bacteria isolated from archaeological vessels and from environmental soil samples based on 16S rRNA gene sequences. Earth samples collected from archaeological vessels-cup and crock, assay cup1, crock1-plating on NA (Nutrient agar), cup2, crock2- inoculation samples on NA (Nutrient agar) after activation of the germination process; cup3, crock3-plating on Streptomyces GYM agar; number after the forward slash indicates the number of analysed bacterial colonies taken from the medium. BG-sample of soil taken in the vicinity of the grave. The species not detected in the control are marked in red.

Number of samples constructed based on sequence similarity	Isolate code	Maximum number of sequenced bases used in alignment.	Phylogenetic affiliation	Sequence identity (%)	The highest 16S rRNA sequence similarity (Genbank)
1	2	3	4	5	6
1	**BG3/3**	391	*Pseudomonas brassicacearum*	99	HQ242755
		391	*Pseudomonas frederiksbergensis*	99	KJ567114
		391	*Pseudomonas lini*	99	HQ242757
		391	*Pseudomonas mediterranea*	99	HQ242760
		391	*Pseudomonas putida*	99	HF545844
2	**crock2/1**	387	*Acinetobacter radioresistens*	99	KP763481
3	**crock1/4, cup1/4, crock3/5, crock3/7, crock3/9, cup3/3, cup 3/5, cup3/7, BG3/1**	370	*Arthrobacter defluvii*	99	KM203781
	crock1/4, cup1/4, crock3/1, crock3/5, crock3/7, crock3/9, cup3/3, cup3/5, cup3/7, cup3/9, BG3/1	370	*Arthrobacter globiformis*	99	KM252929
		372	*Arthrobacter humicola*	99	LK022683
		398	*Arthrobacter oryzae*	99, 100	KR233762
		398	*Arthrobacter oxydans*	99	KR085876
		398	*Arthrobacter pascens*	99, 100	KT239466
		398	*Arthrobacter phenanthrenivorans*	99,100	KR085846
		398	*Arthrobacter scleromae*	99,100	KP739254
		398	*Arthrobacter siccitolerans*	99,100	KP192022
		398	*Arthrobacter sulfonivorans*	99,100	KP192019
4	**BG3/2**	366	*Arthrobacter aurescens*	99	AB741459
		366	*Arthrobacter bambusae*	99	KP860526
		366	*Arthrobacter nitroguajacolicus*	99	HG941862
		366	*Arthrobacter gyeryongensis*	99	JX141781
		366	*Arthrobacter methylotrophus*	99	LN774207
		366	*Arthrobacter ramosus*	99	KF958504
5	**cup2/4**	391	*Paenibacillus alginolyticus*	99	NR_040893

1	2	3	4	5	6
		391	*Paenibacillus chondroitinus*	99	KP203953
		391	*Paenibacillus frigoriresistens*	99	NR_109546
		391	*Uncultured Paenibacillus sp.*	99	KJ191874
6	**BG1/4**	388	*Bacillus pumilus*	100	KT624198
		388	*Bacillus safensis*	100	KP940383
7	**cup1/6, cup 1/7.2, crock3/3, cup3/2, BG1/3**	395	*Bacillus cereus*	98, 99	LC076294
		395	*Bacillus thuringiensis*	98, 99	KP998181
		395	*Bacillus anthracis*	98, 99	CP012519
		395	*Bacillus subtilis*	98, 99	KP986945
	cup1/7.2, crock3/3, cup3/2	395	*Bacillus toyonensis*	98, 99	KM241845
	cup1/7.2, crock3/3, cup3/2, BG1/3	395	*Bacillus mycoides*	98, 99	KM251860
	cup1/6	390	*Bacillus pseudomycoides*	98	HM209758
8	**crock2/1, crock2/5**	391	*Bacillus marisflavi*	99	KJ560874
		391	*Bacillus aquimaris*	99	KP178608
		391	*Bacillus vietnamensis*	99	KC734535
9	**crock3/8, BG1/1**	386	*Bacillus aryabhattai*	99	KR006696
	crock3/8	387	*Bacillus megaterium*	99	KC595869
	BG1/1	386	*Bacillus megaterium*	99	KR822272
10	**BG1/2**	398	*Psychrobacillus psychrodurans*	97	KC354598
		399	*Actinobacterium*	97	EU810854
		399	*Bacillus cereus*	97	KJ922989
11	**cup1/1**	400	*Psychrobacillus psychrodurans*	99	KM036085
12	**cup2/3, BG2/3**	388	*Azorhizophilus paspali*	96,100	LN874287
		388	*Bacillus arbutinivorans*	96,100	KR131862
		388	*Bacillus drentensis*	96,100	KJ589539
		388	*Bacillus fumarioli*	96,100	KC354687
		388	*Bacillus senegalensis*	96, 100	AB795570
		388	*Bacillus thermocopriae*	96,100	KP010244
13	**crock3/10, crock1/2, crock1/6, crock2/2**	427	*Uncultured bacterium*	98,99	AB929995
	cup1/3	414	*Bacillus sp.*	99	LC065250
14	**cup3/10**	416	*Brevibacterium frigoritolerans*	98,99,100	KR085896
		416	*Bacillus megaterium*	98,99,100	KP717948
		416	*Bacillus muralis*	98,99,100	KM678279
		416	*Bacillus simplex*	98,99,100	KP993465
		416	*Bacillus thuringiensis*	98,99,100	KP966473

1	2	3	4	5	6
		416	*Marine bacterium*	98,99,100	KJ814538
		392	*Paenibacillus sp.*	100	KT200444
		416	*Psychrobacillus psychrodurans*	98,99,100	KR088747
15	**cup2/1**	425	*Bacillus muralis*	99	EU977778
	cup2/1, cup2/2, crock3/4	425	*Bacillus simplex*	99	JF683660
	cup2/1, cup2/2, crock2/2, cup1/3, cup2/4, cup1/3, crock3/11, crock3/4, cup3/1, cup3/6, cup3/8, BG1/6	424	*Paenibacillus sp.*	98, 99, 100	KT200444
		424	*Psychrobacillus psychrodurans*	98, 99, 100	KR088747
		424	*Brevibacterium frigoritolerans*	98, 99, 100	KR085896
		424	*Bacillus megaterium*	98, 99, 100	KP717948
	cup1/3.1, cup2/4, cup1/5, crock3/11, crock3/4, cup3/1, cup3/6, cup3/8, BG1/6	416	*Bacillus thuringiensis*	98, 99, 100	KP966473
	cup1/3.1, cup2/4, cup1/5, crock3/4, cup3/1, cup3/6, cup3/8, BG1/6	416	*Marine bacterium*	98, 99, 100	KJ814538

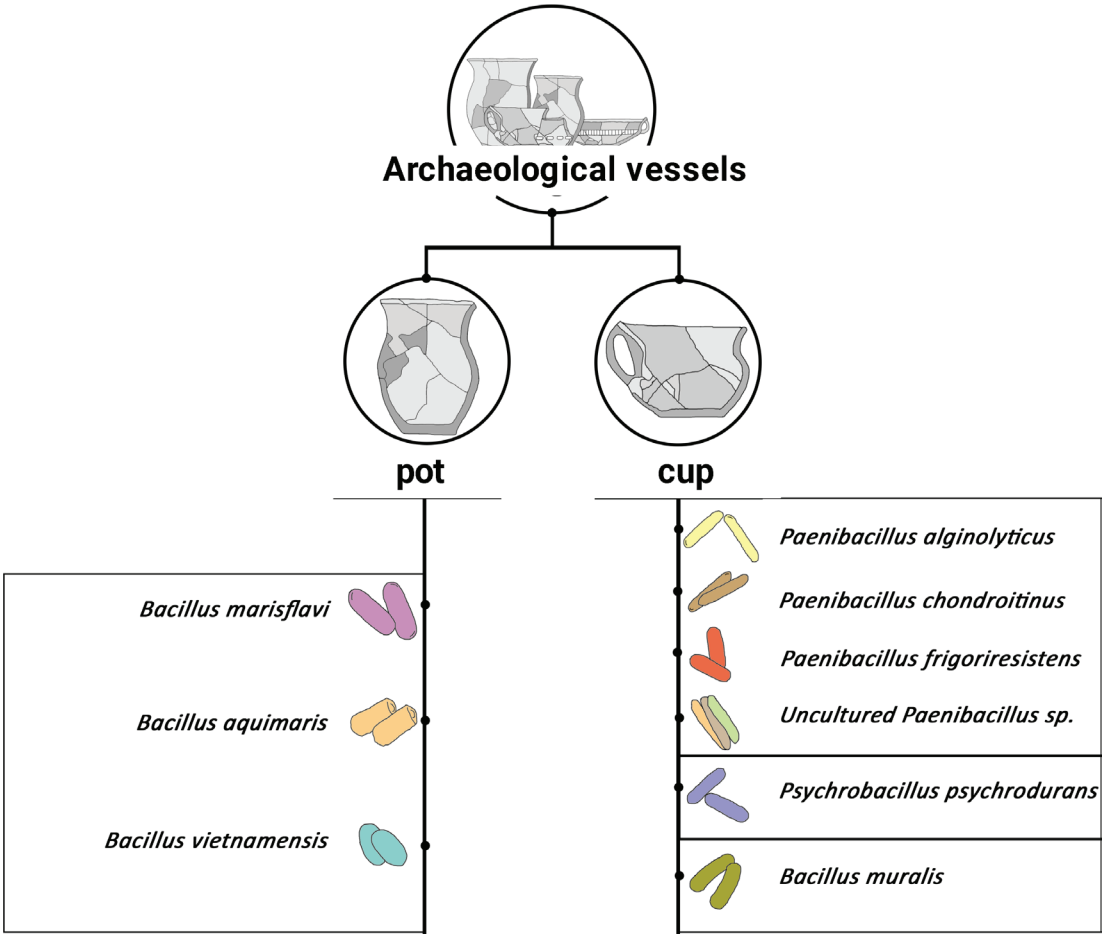

Fig. 3. Site-specific culture-based bacterial communities in a) crock; b) cup sampling locations (figure created with Biorender.com; prepared by L. Potocki).

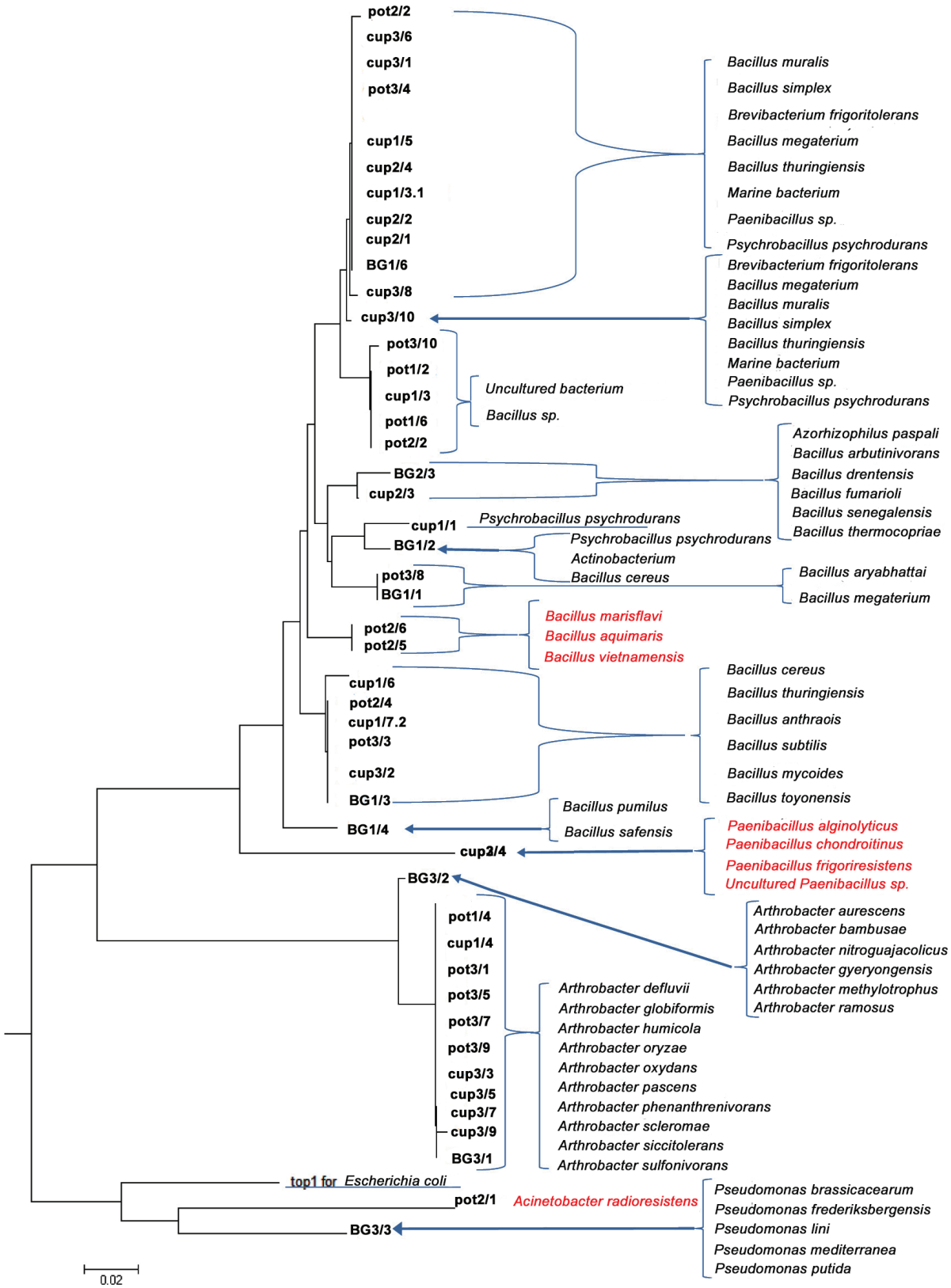

Fig. 4. Phylogenetic relationships of bacteria isolated from archeological vessels and from environmental soil samples based on 16S rRNA gene sequences. Scale bar represents 0.02 substitutions per nucleotide position. *Escherichia coli* strain Top 10 is used as outgroup. The species not detected in the control are marked in red. Earth samples collected from archaeological vessels-cup and crock, assay cup1, crock1-plating on NA (Nutrient agar), cup2, crock2-plating on Streptomyces GYM agar, cup3, crock3- inoculation samples on NA (Nutrient agar) after activation of the germination process; number after forward slash indicates number of analyzed bacterial colony taken from the medium. BG-sample of soil taken in the vicinity of the grave (prepared by L. Potocki).

ence of this genus is dominant in the microbiome of archaeological vessels. Therefore, it seems reasonable to ask whether the isolated bacteria are descendants of ancient bacteria that may have thrived in the nutrient-rich medium found in the vessels. According to a well-established view in archaeology, it is assumed that vessels discovered in graves (if they had no other function, such as being used as urns filled with bones), including additional ceramic grave goods, were originally filled with food (Malinowski 1985). Despite the lack of evidence for the presence of organic compounds in vessels from Dębina, this relationship was confirmed in another research work, where the presence of potassium (K) compared to the values of sodium (Na) was considered an indicator of the presence of organic substances; also the case in the analyzed sand samples that fill the vessels discovered in graves (Bulska and Wróbel 1992). The approach presented here may be promising in determining whether certain bacterial taxa can be indirect evidence indicating that the above vessels were filled with food, cereal seeds or beverages (wine, oil, milk, honey) deposited in an act of worship (Alippi *et al*. 2002; De Graaf *et al*. 2006). Analyzes of grave fillings and traces on the inner walls of vessels carried out at the late Bronze Age and early Iron Age cemetery in Cottbus (Brandenburg, Germany) using a mass spectrometer and gas chromatograph revealed traces of fatty acids, monostearates and sterols in one vessel. The analyzed vessel was originally filled with a liquid substance containing fat (Hofmann *et al*. 2013). Other analyses (including material from other periods), using atomic absorption spectrometry (with indicators – the presence of copper in the samples and the potassium to sodium ratio), also indicate that vessels placed in graves were filled with organic products of animal origin (Bulska *et al*. 1996). Furthermore, analysis of the sand from vessels from the Lusatian culture cemetery in Maciejowice (Mazovia voivodeship) also revealed the presence of organic substances (Mogielnicka-Urban 1992).

Does the microbiological profile obtained in our study, in which the genus *Paeanibacillus* as well as other sporulation species belonging to the genus *Bacillus* dominate in the spore fraction, constitute an indication of the presence of organic substances in vessels from the grave in the Bronze Age cemetery in Dębina? There are confirmations that the use of bee products by communities at that time were used in the production of bronze objects. For example, bronze objects were cast using the "lost wax" method, and required large amounts of beeswax (Dąbrowski 1992). There are also data that indicate that honey in funerary rituals had a symbolic meaning, signifying rebirth and representing a sacrifice to certain deities (Cirlot 2007). Therefore, its presence in funerary rituals should not be surprising, especially in the Bronze Age, when its "abundance favoured ritual use" (Mierzwiński 2012). In the culture of the Urnfield cultural circle, the custom of libation feasts over the grave of the deceased became widespread in the late Bronze Age and Early Iron Age, as evidenced by significant archaeological evidence, the number and assortment of small vessels in graves. Consumption of a honey-based drink seems to have been a common ritual and cases of filling bronze grave vessels with honey are also known (Rösch 1999; Mierzwiński 2012). The fact that bee products were used in the production of bronze objects and funerary rituals in the Bronze Age further supports the hypothesis that the vessels from Dębina may have contained honey. Honey is a sticky substance that can leave a residue on surfaces even after it has been removed. These residues can be ideal breeding grounds for bacteria such as *Paenibacillus* and *Bacillus*. Data indicates that contaminated honey may serve as an environmental reservoir of *Paenibacillus* larvae spores. Their detection in archaeological vessels may provide evidence of organic residues, such as fermented beverages like beer and mead-honey wine (Lindström *et al*. 2008). Despite the lack of detection of *Paeanibacillus larvae* and *Paeanibacillus alvei* in our research, the identification of species of the genus *Bacillus* (*B. megaterium*, *B. cereus*, *B. brevis*, *B. coagulans*, *B. subtilis*) does not necessarily indicate their environmental origin. The literature has reported that these species were widespread, as were *Brevibacillus* and *Virgibacillus* from bee nests (Gilliam 1985; Sáez-Nieto *et al*. 2017). Thus, the species composition of the control samples may not always reflect the environmental isolates but can also provide valuable information regarding the presence of species common to test and control samples.

The microbiological analysis also showed the presence of *Bacillus muralis*, which has been detected on the murals of churches, and decorated entrances to the graves (Heyrman *et al*. 2005*)*. In this case, the identification of the species in the microflora of the soil sample taken from the cup may suggest the presence of dyes that might have come from the decorated dish.

In order to confirm the research hypothesis presented in the paper, further research could include conducting chemical analysis of the vessels to search for the residue of honey or other organic substances. Organic sediment analysis of ceramics is a relatively new research method that is gaining popularity among archeologists. This method allows the detection and identification of

organic residues, such as fats, proteins, carbohydrates, and DNA, which can be preserved in sediments on the surface or inside ceramic vessels (Evershed 2008; Mayyas *et al*. 2013; Bondetti *et al*. 2020).

In summary, we have shown that the characterization of DNA sequence haplotypes of soil bacteria derived from endospores can provide an additional source of information in the archaeological discovery of past funeral rituals in various human populations that used food during their ceremonies. The proposed method also has some advantages over other analytical methods such as eAAS, GC-MS and HPLC, because it is based on the analysis of bacterial composition, which depends strictly on the availability of specific organic and inorganic compounds, e.g., derived from animal and food pigments and human bodies.

Moreover, the presented method of using degenerate primers to study the diversity of microorganisms obtained from archaeological samples may be an alternative to more expensive metagenomic analyses.

4. Conclusions

The conducted microbiological analysis showed a variable composition of spore bacteria collected from archaeological vessels from a grave. Detection of taxa of the genus *Paeanibacillus* or *Bacillus* may be evidence of anthropogenic rites of burying the dead in the Tarnobrzeg Lusatian culture. The presented bioindication of microorganisms may be a valuable new method supplementing traditional archaeological methods. A very interesting development of the analysis was the isolation of a species of *Bacillus muralis*, often detected as a component of the heterotrophic microflora present on the surface of ancient frescoes or wall paintings. The presented approach of using degenerate primers to study microbial biodiversity in archaeological samples may be an alternative to more expensive metagenomic analysis.

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