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BRIEF REPORT

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Revealing human impact on natural ecosystems through soil bacterial DNA sampled from an archaeological site

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Abstract

Human activities have affected the surrounding natural ecosystems, including belowground microorganisms, for millennia. Their short- and medium-term effects on the diversity and the composition of soil microbial communities are well-documented, but their lasting effects remain unknown. When unoccupied for centuries, archaeological sites are appropriate for studying the long-term effects of past human occupancy on natural ecosystems, including the soil compartment. In this work, the soil chemical and bacterial compositions were compared between the Roman fort of Hegra (Saudi Arabia) abandoned for 1500 years, and a preserved area located at 120 m of the southern wall of the Roman fort where no human occupancy was detected. We show that the four centuries of human occupancy have deeply and lastingly modified both the soil chemical and bacterial compositions inside the Roman fort. We also highlight different bacterial putative functions between the two areas, notably associated with human occupancy. Finally, this work shows that the use of soils from archaeological sites causes little disruption and can bring relevant information, at a large scale, during the initial surveys of archaeological sites.

INTRODUCTION

In recent decades, human activities including urbanisation, industrialisation and the growing demand for biological and mineral resources have increased pressure on terrestrial ecosystems (Huang et al., 2010; McFadden et al., 2022). These activities often disrupt ecosystems, locally or remotely, resulting in changes in the biodiversity and the composition of macro- and micro-organism communities (Addison et al., 2019; Cantera et al., 2022; Fenoglio et al., 2020; Thiele-Bruhn et al., 2012). Soil accounts for a large proportion of the biodiversity of terrestrial ecosystems and plays a key role in their functions and services, but the relationships between the microbial composition and the soil functions in ecosystems are still poorly understood (Bardgett & van der Putten, 2014).

Human occupation is known to deteriorate soils (compaction, pollution, etc.), resulting in changes in soil properties and chemical composition (Gadd, 2010; Pelosi et al., 2021). In turn, these abiotic changes often modify the soil microbial composition, resulting in changes in soil functions (e.g., nitrogen and carbon cycles) (Epp Schmidt et al., 2017; Gallas & Pavao-Zuckerman, 2022; Wang et al., 2017). Studies of the impact of soil human occupation usually focus on short-term (<10 years) (de Vries et al., 2006; Yu et al., 2022) and medium-term (<100 years) (Addison et al., 2019; Boot et al., 2016; Wu et al., 2021) effects, mainly in temperate climates. Therefore, little is known about the long-term effects (>1000 years) of soil human occupation, especially in poorly studied areas such as deserts (Guerra et al., 2020).

Hot deserts cover about 13.5 million square kilometres and are currently expanding due to global warming (Makhalanyane et al., 2015; Wu et al., 2022). The two largest hot deserts in the world are located in northern Africa (the Sahara Desert; 9.2 million square kilometres) and the Arabian Peninsula (the Arabian Desert; 2.3 million square kilometres) (Cook & Vizy, 2015). These hot desert ecosystems are not excluded from human occupation and are particularly prone to rapid and large-scale urbanisation whose long-term consequences are unknown (Burt & Bartholomew, 2019). Human occupation of these hot ecosystems is not new, and important civilisations including Egyptian, Persian, Nabataeans and Romans have colonised these areas for more than five millennia (Lichtheim, 2019; Mathisen, 2018; Sansone, 2016). During this long period, many cities were built to house, defend, feed, entertain and administer the populations, and some were then abandoned before being rediscovered (e.g., the Nabataean civilisations in Petra in Jordan, and Hegra in Saudi Arabia) (Nehmé, 2020; van der Steen, 2019). What is certain is that the construction and occupation of these cities in the past had a major impact on the biodiversity and the functions of these natural ecosystems. Their abandonment for centuries, and the resulting absence of human activities over a long period, enabled the surrounding biodiversity to gradually recolonise the sites. These archaeological sites are therefore appropriate to study the long-term impact of urbanisation and to assess the ability of the surrounding biodiversity to recolonise sites after centuries of human absence.

In this study, we focus on the archaeological site of Madâ'in Sâlih (ancient Hegra), which is located 20 km north-east of the city of al-Ula in Saudi Arabia and covers approximately 3 km^2 (Nehmé, 2021). A few years after the Nabataean kingdom was annexed by the Romans, in AD 106, a fort was built in the southern part of Hegra as a base for the military garrison responsible for protecting Roman interests in that part of the eastern frontier (Fiema, 2018). Ongoing Saudi–French excavations have revealed that the Roman fort was probably militarily abandoned around the end of the third century or the beginning of the fourth century, and completely abandoned at the beginning of the fifth century. There is no evidence of more recent occupation, so no significant human activity has occurred at the site for at least 1500 years (Fiema, 2018). In the middle of the 20th century, a metal fence was installed around the ancient site of Hegra to protect it from intrusion. This Roman fort, therefore, provides a good opportunity to examine both the long-term impact of past human activities (i.e., urbanisation and related human activities) and the ability to surround biodiversity to recolonise the site after centuries of human absence.

In hot desert ecosystems, vegetation is sparse and the soil is often bare resulting in low aboveground biodiversity. Several archaeological studies have shown that long-term human occupancy modified the relative abundance of chemical elements, including Ca, P, S, Fe, Sr, Zn, Cu, Sn and/or In (Berger et al., 2019; Williams et al., 2020). The objectives of this study were consequently to assess the impact of around 400 years of human occupancy of this Roman fort, and of its subsequent abandonment for 1500 years, on the chemical and the bacterial compositions of the soils compared with soils in surrounding preserved areas (i.e., devoid of archaeological remains). A total of 156 soil samples were collected inside and outside the fort and compared to test our ability to confirm the presence of the fort based on their chemical and bacterial compositions (using a 16 rRNA meta-barcoding approach). Putative chemical and bacterial markers of human occupancy were then identified and linked to putative past human activities. The consequences of changes in the chemical composition of soil on both the composition and the putative functions of the bacterial communities were also tested (inferred from the 16S rRNA sequences). Our results show that studying the bacterial composition of soil provides relevant additional information on putative human activities in the fort and vicinity, to that

provided by chemical analyses. Our results also highlight the fact that urbanisation can still have a profound and lasting impact on natural ecosystems even after centuries of human absence.

EXPERIMENTAL PROCEDURES

Architecture of the Roman fort and collection of the soil samples

The Roman fort studied here is an ancient military installation built in the southern part of Hegra, which, following annexation of the Nabataean Kingdom in 106 AD, found itself in the eastern frontier zone of the Roman Empire. The remains of the fort are located on a plateau at the bottom of a hill, which forms the eastern limit of the fort (Figures S1 and S2). Of particular interest here is the southern perimeter wall (1.3–1.4 m wide and 65 m long) constructed of stone. The southern wall ends in a corner tower from which another wall continues northward forming the western limit of the fort. The interior of the fort includes the eastern wing comprising rooms, aligned north–south, which most probably served as the barracks (RF_079 to RF_081) (Figure S3). In the southwestern part of the fort are remains of a building, which abutted the southern wall. Some of the rooms and spaces in the building have been completely or partially excavated. The whole building can be conveniently divided into three areas, first, a space containing the southern gate into the fort (RF_089 to RF_097), second, a space that is a room containing two stone basins and third, a space (RF_98 to RF_103) (Figures S1 and S3) that included at least two rooms. Judging by the presence of quantities of pottery and bones, as well as the two stone basins discovered during the excavations, the whole building was probably used as a food processing and storage area, but may also, at least partially, have been used to store other material or as a dwelling space (Supporting Information). Between the southwestern rooms and the eastern barracks is an open area (RF_078, RF_082 and RF_084 to RF_087), which contains two distinct rooms (RF_083 and RF_088, respectively) whose function is unknown. A total of 156 soil samples were collected inside and outside the fort. Seventy-seven soil samples were collected along the southern wall and in an area extending a distance of 6 m from it (RF_EXT; sample numbers 1–77). Twenty-six soil samples were collected inside the Roman fort in the different buildings and outdoor spaces (RF_INT; sample numbers 78–104). Finally, 52 soil samples were collected along two transects: 26 along the first transect located about 250 m from the southern wall, to the north of the fort (hence inside the ancient city; [TR_INT; sample numbers 105–130]), and 26 along a second transect located about 250 m from the southern wall, to the south of the fort (thus outside the ancient city, and where no traces of human activities were detected; TR_EXT; sample numbers 131–156) (see Figures S2 and S3). Each sample comprised 200 g of soil collected at a depth of 15–20 cm (the 15 cm was carefully removed to collect only this 15–20 cm soil layer), sieved at 2 mm and conserved in sterile pots, for both the chemical and the molecular analyses. In the Roman fort site, in Saudi Arabia, the soil remains dry (no trace of water) and hot (around 20–30°) at 15–20 cm depth, all year long (except during the rare rainfall; 1–2 times per year), and therefore we chose to not froze the samples before molecular analyses to keep them in the same conditions than the field.

Soil chemical analyses

The pH and the chemical composition of all 156 soil samples were measured (Table S1). pH was measured using a pH meter (pH Meter Knick 766) in 1:5 v:v of deionised water (i.e., pH_H_2O). The relative quantification of soil atomic elements from magnesium (Mg) to Uranium (U) was performed by x-ray fluorescence (XRF) using a portable device (XRF S1 Titan, Bruker). To improve soil compaction, each soil sample was diluted in 6:1 v:v of Spectroblend (SCP Science), in triplicate, and pressed under 20 tons (manual press, Specac) in aluminium capsules (AluCaps 38 mm Flared, SCP Science). Three shots of XRF per replicate were performed giving a total of nine shots per soil sample (GeoExploration mode with the following settings: phase 1, 30 s; phase 2, 30 s and phase 3, 45 s). The nine measurements were then averaged and calibrated according to the limit of optical detection of each atomic element (provided by Bruker).

Soil DNA extraction

Before DNA extraction, 5 g of each soil sample was diluted in 20 mL of sterile Milli-Q water, and shaken on a turntable for 30 min at 150 rpm. Then, the mix was decanted for 15 min on the bench, and the supernatant was collected and centrifuged at 12,000*g* for 5 min. Finally, the pellet was isolated for DNA extraction. The total DNA was extracted using the FastDNA SPIN for soil kit (MP Biomedicals) following the manufacturer's instructions. DNA assays were performed in microplates with PicoGreen (Quant-IT™ PicoGreen, Invitrogen) using a Spark device (Tecan).

PCR amplification

DNA concentrations were normalised between samples and three independent PCR amplifications were

performed for each sample (each with 1 ng of DNA), using the Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) and the primers F479 (5′ CAGCMGCYGCNGTAANAC 3′)/R888 (5′ CCGYCAATTCMTTTRAGT 3′) to amplify the bacterial hypervariable 16S rRNA V3V4 region (Terrat et al., 2015). At this first PCR step, each of the forward and reverse primers contains specific barcodes to tag each sample separately. The PCR conditions were as follows: initial denaturation for 10 min, 35 denaturation cycles at 94°C for 10 s, annealing at 55°C for 10 s and extension at 72°C for 10 s, with a final polymerisation extension at 72°C for 7 min. All reactions were performed in triplicate and were then pooled before deposition on 2% agarose gel, with the corresponding negative control, for quality control of the amplifications. From the 156 soil samples, only 148 were successfully amplified (excluding the RF_061, RF_062, RF_067, RF_068, RF_073, RF_097 and RF_100 from the bacterial analysis; Figure 2; Table S2).

Preparation of amplicon libraries

The 16S amplicons were purified with AMPure XP beads (Beckman Coulter). Samples containing amplicons were placed on a magnetic rack with a 1:1 ratio of AMPure XP for each PCR product. The magnetic beads were rinsed twice with ethanol 1:1 v:v before final elution in 70 μL of EB buffer (Qiagen). The concentrations of the purified amplicons were then measured with PicoGreen, and an equimolar pool (50 ng per sample) was prepared. Each pool was finally purified twice, quantified with PicoGreen and eluted in 40 μL of EB buffer.

Illumina MiSeq high-throughput sequencing and data processing

MetaFast library preparation and sequencing were performed on an Illumina 2 × 250 bp MiSeq platform by Fasteris SA, including the addition of Illumina adaptators (www.fasteris.com). A pipeline based on VSEARCH (Rognes et al., 2016) and available on GitHub (https://github.com/BPerezLamarque/Scripts/) was used for data processing (Petrolli et al., 2021). Briefly, after merging paired-end reads (fastq_mergepairs function, default parameters), we did a quality check and removed merged reads with more than two errors in alignment. Merged reads were demultiplexed (assigned to their respective sample based on tagged primers) with 0 error accepted in primers or rag sequences, using cutadapt (Martin, 2011). Chimeras were removed de novo (uchime3_denovo option of VSEARCH) and the taxonomic assignment of the Amplicon Sequence Variants (ASV) was performed (usearch_global option, default parameters) using the SILVA 138.1 database (Quast et al., 2013). Contaminants were filtered out of the ASV tables based on comparison with amplified negative controls using the R package DECONTAM (prevalence method) (Davis et al., 2018). Only ASVs with long sequences (>200 bp), assigned to the bacterial kingdom, acceptable abundance (≥50 reads), and acceptable prevalence (≥3%, i.e., present in at least four samples), were kept for downstream analyses, giving a total of 3161 clean bacterial ASVs.

Statistical analyses

All the statistical analyses were performed with R software. The XRF measurements provide the relative abundance of atomic elements from Mg to U, which results in a compositional dataset including some correlated variables and non-detected elements (values = 0). To simplify the analysis, elements that were not detected were removed, and the dataset was centered-log ratio (clr) using the package 'compositions' (van den Boogaart & Tolosana-Delgado, 2008) to open data from the closure effect. Principal component analysis was then performed to identify correlated variables using the package 'FactoMineR' (Lê et al., 2008). Eight variable representatives of the chemical dataset were selected (Mg, Si, P, S, Ca, Fe, Sr and Sn). It should be noted that the pH is closely correlated with Si content and the pH measurements were consequently removed from the dataset for further analyses. Unsupervised clustering was then performed on the chemical clr dataset, using the HCPC function from the package 'FactoMineR', resulting in the three different clusters of soils (named 'soil clusters 1, 2 and 3'). Permutational multivariate analysis of variance (PermANOVA, p-value < 0.001) was performed to test the significance of the three soil clusters using the 'adonis' function (with 999 permutations and the Euclidean method) from the 'vegan' package, and a Kruskall–Wallis test followed by a posthoc pairwise test was performed to test the significance of each chemical element in the three soil clusters, using the 'dunn.test function (with Bonferroni correction) in the 'dunn.test' package (p value < 0.05).

For the bacterial dataset, the Illumina MiSeq high-throughput sequencing yielded 1,614,279 cleaned reads from the 148 soil samples with an average of 10,907 reads per sample (range 2449–40,594). The sequencing dataset was studied using the microeco (Liu et al., 2021) and the phyloseq (McMurdie & Holmes, 2013) packages in R, and all further analyses were performed using the relative abundance of the ASVs, except alpha-diversity. To identify the four bacterial clusters corresponding to significant differences between the bacterial compositions of the soil samples,

the Bray–Curtis dissimilarity index between each pair of samples was first calculated, and the same unsupervised clustering method was applied as the one used for the chemical dataset. Significant differences between the four bacterial clusters were tested using a permutation test (PermANOVA; p-value < 0.001). The 'SpatialEpi' package was used to calculate the species contribution to beta diversity (SCBD). To study the level of diversity within each bacterial cluster, the dataset was rarefied to 2500 reads in each sample, and the Chao1 (richness) and the Simpson (evenness) indexes were calculated, and compared using ANOVA (p-value < 0.05). Differential abundances of the taxonomic orders and the functional groups were identified using the 'Aldex2' package. First, the functional inference of each ASV was made using the FAPROTAX database (Louca et al., 2016), and the functional redundancy was then calculated for each taxonomic order. A Kruskal–Wallis test with Benjamini–Hochberg correction was applied to test the significance of the differential abundances (p-value < 0.05). The percentage of variance for both the bacterial abundance and the bacterial functional groups, explained by the eight representative chemical elements, was calculated by redundant analysis (RDA) using the 'BiodiversityR' package. As both the abundance and the functional datasets are compositional, a Hellinger transformation was performed before the RDA using the 'vegan' package. A random independent permutation test and Holm correction were then applied to identify significant chemical elements associated with differential bacterial abundances and functions (p-value < 0.01). Within each functional group, a Kruskal–Wallis test with Bonferroni correction followed by a post-hoc pairwise test was performed to test for significant differences between the four bacterial clusters (p-value < 0.05).

RESULTS

Changes in the chemical composition of the soil were strongly associated with the presence of the Roman fort

To judge whether the establishment of the fort and its occupancy for about 400 years had a lasting impact on the chemical composition of the soil in this dryland ecosystem, we assessed the relative abundance of chemical elements using x-ray spectrometry (see Experimental procedures and Table S1). First, we tested our ability to confirm the location of the fort based only on changes in the chemical composition of the soil. For that purpose, we used an unsupervised clustering method that resulted in three soil clusters corresponding to significant differences in their chemical compositions (hereafter 'soil cluster 1, 2 and 3'; Figure S4). Soil Cluster 1 differed significantly from soil Cluster 3 in that the relative abundances of Si and Mg were significantly higher in Cluster 1, whereas the relative abundances of the Ca, S and Sr were higher in Cluster 3. Soil Cluster 2 differed from Clusters 1 and 3 in its higher relative abundances of P and Fe and its lower relative abundance of Sn. The three soil clusters were then mapped on the plan of the Roman fort (Figure 1). The majority of soil Cluster 1 was located outside the fort, whereas Cluster 3 was found mainly inside the fort, along the southern wall, plus a few patches along the interior transect (TR_INT). These results therefore identify a strong link between the changes in the chemical composition of the soil (i.e., higher relative abundances of Ca, S and Sr) and the presence of the Roman fort. They also suggest the presence of putative buried buildings in the TR_INT (corresponding to the ancient city; see Experimental procedures).

The increase in the relative abundance of specific chemical elements may be due to the presence of buried metal artefacts (e.g., Zn, Cu, Fe, Sn, among others), or to human activities (e.g., Ca, P, Sr for food storage and processing, and S for fire/cooking) (see Introduction). We consequently searched for these specific chemical signatures in the soil samples. High relative abundances of In (37–443 ppm) were found in 12 soil samples located along the southern wall (RF_005 to RF_007, RF_061, RF_067, RF_074, RF_084, RF_085, RF_087), in a room in the eastern wing (RF_081) and in the TR_INT (RF_110 and RF_111) (Table S1). These unexpectedly high relative abundances of indium were also linked with the highest relative abundances of copper and tin (>150 ppm) (average [Cu] = 36 ppm; average [Sn] = 59 ppm) of all the soil samples. These results suggest the presence of metal elements in the southern wall (which may have since been removed), and the use of metal objects in some rooms in the eastern wing and the putative buried buildings in the eastern part of the TR_INT. High relative abundances of Ca (>7%) (average [Ca] = 2.5%), Sr (>1500 ppm) (average [Sr] = 435 ppm) and P (>2500 ppm) (average [P] = 1223 ppm) were detected in all three parts of the building abutting the southern wall (RF_091, RF_092, RF_095, RF_096, RF_098, RF_099, RF_100), suggesting the building was probably used to store and prepare food, in agreement with the results of excavations already performed in this area (see Experimental procedures). The same chemical signature was also detected in a soil sample collected close to this building, in an outdoor area of the Roman fort (RF_087). In this sample, the high relative abundance of Ca (8.6%), Sr (2204 ppm) and P (2,427 ppm) is also associated with a high relative abundance of S (11.9%; average [S] = 1.6%), suggesting a place where food was cooked. Interestingly, another soil sample collected outside the fort (RF_053) was the only soil sample with a chemical signature

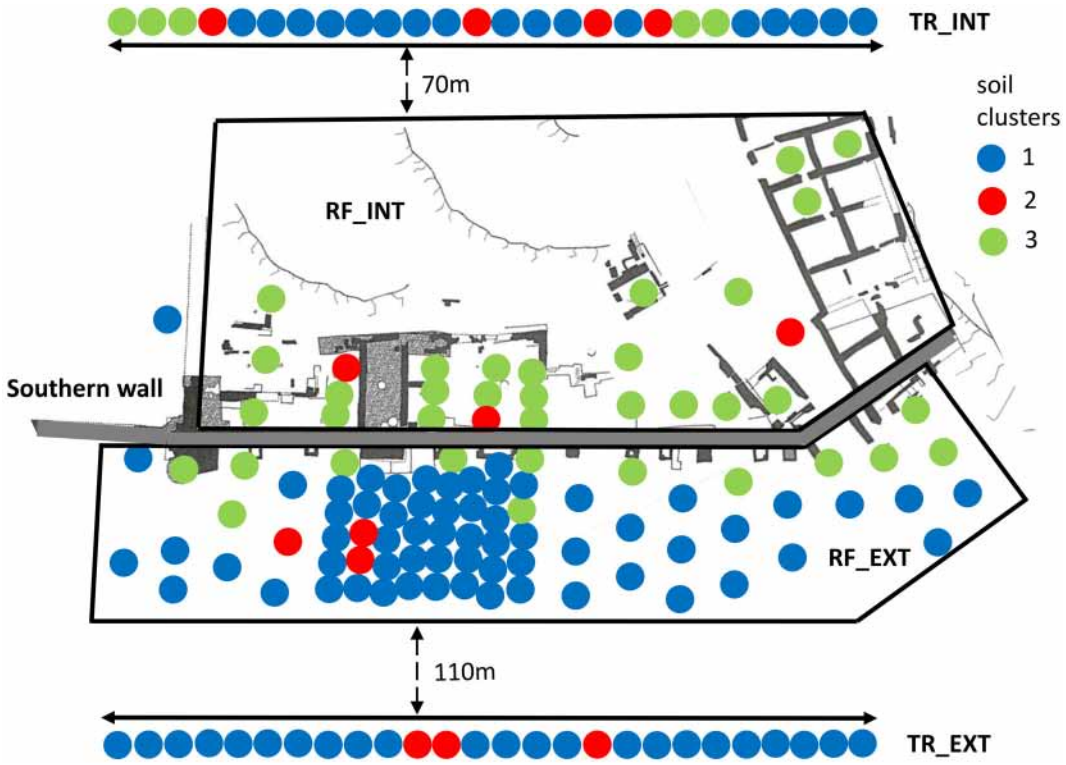

FIGURE 1 Chemical composition of the soil samples collected in and around the Roman fort. Drawing of the fort including the location of the 156 soil samples collected. Each soil sample was assigned to a cluster based on its chemical composition (see Experimental procedures). RF_EXT, exterior of the fort; RF_INT, interior of the fort; TR_INT, interior transect; TR_EXT, exterior transect.

similar to that of the RF_087 (Ca = 7.8%, Sr = 2060 ppm, P = 1700 ppm, S = 8.9%), suggesting a place where food remains might be thrown away after cooking.

Soil bacterial community composition was impacted by the presence of the Roman fort

Like with the chemical soil analyses, we tested our ability to confirm the presence of the Roman fort based on differences in the bacterial composition of the soil samples. For that purpose, total DNA was extracted from the 156 soil samples and high-throughput sequencing of the high variable V3V4 region of the 16S rRNA was performed (for sequencing and statistical details, see Experimental procedures). Overall, the sequencing dataset showed that the soil samples were dominated by Proteobacteria, Actinobacteria, Firmicutes, Chloroflexi, Bacteroidota and Gemmatimonadota, but significant differences in bacterial composition were observed between samples (Figure S5a). The Bray–Curtis dissimilarity index was calculated between each pair of samples. Based on these indexes, an unsupervised clustering method was then performed that resulted in four significantly different clusters, corresponding to four significantly different bacterial compositions of the soil (hereafter 'bacterial cluster 1, 2, 3, and 4'; see Experimental procedures; Figure S5b,c). Mapping the four bacterial clusters on the plan of the Roman fort revealed a strong link between the bacterial composition of the soil and the presence of the Roman fort (Figure 2). Bacterial Cluster 1 was mainly located outside the Roman fort (i.e., RF_EXT, TR_INT and TR_EXT), whereas bacterial Cluster 3 was mainly located inside the fort, particularly inside the multi-roomed building that abutted the southern perimeter wall. Bacterial Cluster 2 was mainly found along the southern wall and in some patches along the interior transect. Interestingly, bacterial Cluster 4 was found outdoors, close to the building abutting the southern wall (RF_087 to RF_090), but also on the western part of the exterior transect, suggesting there was a link between these two distant areas. This result differs from the chemical data, which indicated no difference along the exterior transect.

Two taxa dominated the bacterial composition of soils sampled inside the Roman fort

Next, we analysed differences in the composition and the diversity of the four bacterial clusters (see

FIGURE 2 Bacterial composition of the soil samples collected in and around the Roman fort. Drawing of the fort including the location of the 156 soil samples collected. Each soil sample was assigned to a cluster based on its bacterial composition (see Experimental procedures). Soil samples with no amplicon were not assigned to a cluster (NA). RF_EXT, exterior of the fort; RF_INT, interior of the fort; TR_INT, interior transect; TR_EXT, exterior transect.

Experimental procedures). Among the 12 most abundant orders in the entire dataset, bacterial Cluster 1 was significantly enriched in Thermomicrobiales, Rubrobacterales, Gaiellales and Solirubrobacterales; bacterial Cluster 2 in Cytophagales, Longimicrobiales, Flavobacteriales, Bacillales and Paenibaciallales; bacterial Clusters 3 in Burkholderiales and Rhizobiales; and bacterial Cluster 4 in Pseudomonadales (Figure S6a,b). Intra-diversity indexes were then used to assess the difference in bacterial diversity within each cluster. Bacterial Cluster 1 had higher richness (Chao1 index; Figure S6c) and higher evenness (Simpson index; Figure S6d) than the three other clusters, while bacterial Cluster 3 had both the lowest richness and the lowest evenness. Bacterial Cluster 2 had low richness and high evenness, and bacterial Cluster 4 had intermediate richness and evenness. These results show that bacterial Clusters 3 and 4 differed dramatically from Clusters 1 and 2 and that only a few taxa dominated the composition of bacterial Clusters 3 and 4. They also suggest the presence of a gradient of bacterial diversity from outside the Roman fort (i.e., bacterial Cluster 1; high diversity; except for the west part of the exterior transect) to the inside (i.e., bacterial Clusters 3 and 4; low diversity), with intermediate diversity along the southern wall and in patches along the interior transect (i.e., bacterial Cluster 2; intermediate diversity).

To determine which taxa dominated bacterial Clusters 3 and 4, and how much they contributed to the total bacterial diversity of the area, we analysed the SCBD. The results revealed that the two first ASV of the bacterial dataset correspond to a *Ralstonia insidiosa* and a *Pseudomonas* sp. (Table S2). These two ASVs accounted for respectively, 83% (SD ± 16%) and 74% (SD ± 14%) of the relative abundance of bacterial Clusters 3 and 4, and for 17% (7% and 10%, respectively) of the beta-diversity of the whole dataset (Figure S7), thereby confirming that only two taxa dominated the bacterial composition of most soil samples collected inside the Roman fort.

Increase in the number of taxa related to putative functions associated with humans and animals inside the Roman fort

We found that two species dominated the bacterial composition of the soil sampled inside the Roman fort, in contrast to outside the fort where relatively high

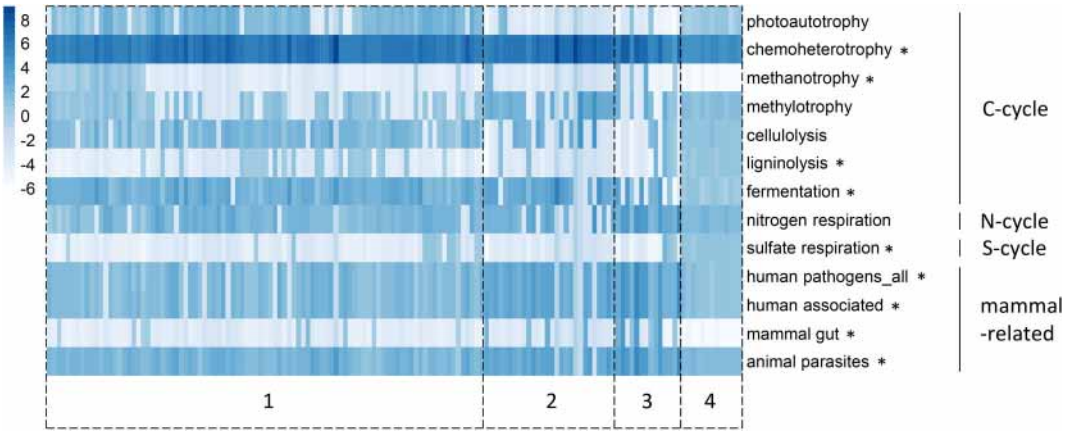

FIGURE 3 Relative abundance of the 13 functional groups found in the soil samples collected in and around the Roman fort. Heatmap representing the centred log ratio (clr) of the relative abundance of the 13 functional groups in the soil samples related to the carbon cycle (C-cycle), the nitrogen cycle (nitrogen respiration), the sulphur cycle (S-cycle) and the human- and animal-associated functions (mammal-related). The numbers (1–4) correspond to the four bacterial clusters. Stars represent significant differences between the four bacterial clusters (Kruskal–Wallis with Benjamini–Hochberg correction; p-value < 0.05; see Experimental procedures).

bacterial diversity was found. To test whether these differences in composition were associated with changes in the putative functions, we compared the proportions of ASV associated with nine metabolic functional groups and four human- and animal-associated functional groups among the four bacterial clusters (see Experimental procedures). A total of 687 ASVs from the whole dataset (21% of all ASVs) were successfully inferred with at least 1 of the 13 functional groups, representing 784,081 reads (48% of all the reads). A shift in carbon energy sources (increase in ligninolysis instead of chemoheterotrophy, methanotrophy and fermentation), as well as an increase in sulphate respiration, were observed in bacterial Cluster 4 compared to the three others (Figure 3; Figure S8). The increase in sulphate respiration is in accordance with the high sulphur content detected in the area outdoors but still within the boundaries of the Roman fort (i.e., RF_087) where bacterial Cluster 4 was located, but not with the western part of the exterior transect where the amount of sulphur was low (Figure S8; Table S1). An increase in the number of taxa associated with humans and animals was detected in bacterial Clusters 2 and 3 (located inside the Roman fort, along the southern wall, and in the patches along the interior transect, TR_INT) in agreement with four centuries of human occupancy of the Roman fort. ASV_0002, which dominated bacterial Cluster 3 located inside the fort, is related to *Ralstonia insidiosa* (Burkholderiales), and associated with a human bloodstream infection (Fang et al., 2019). In addition, five other taxa were significantly more abundant in bacterial Clusters 2 and/or 3, and are related to *Burkholderia mallei* (Burkholderiales), *Achromobacter* sp. (Burkholderiales), *Escherichia coli* (Enterobacterales), *Staphylococcus aureus* (Staphylococcales) and *Mobiluncus curtisii* (Actinomycetales) (Table S3).

The chemical composition of the soil explains the taxonomic diversity better than the putative functions

The differences observed in the composition and the putative functions of the bacterial clusters inside the Roman fort compared to the outside could be related to edaphic conditions (e.g., soil properties) and/or to direct or indirect biotic interactions (e.g., bacterial competition, past human occupancy, etc.). To determine the extent to which the differences in soil conditions explain the composition and the putative functions of the bacterial clusters, redundancy analyses (RDA) were used for variance partitioning (see Experimental procedures). The chemical composition of the soil explained 33% of the total variance of the bacterial diversity at the order level (R^2 adjusted; Figure 4A), where Si, Mg, P, S and Ca are significant (with respectively 6.3%, 3.6%, 1.8%, 1.3% and 0.7% of the explained variance; Figure 4B). The first RDA axis (25.2% of variability) opposes soils enriched in Bacillales, Paenibacillales, Rubrobacterales, Solirubrobacterales, Thermomicrobiales and Gaiellales (bacterial Clusters 1 and 2), mainly found in soils with high relative abundances of Si and Mg (soil Cluster 1), and soils enriched in Cytophagales and Burkholderiales (bacterial Cluster 2 and 3, respectively), mainly found in soils with high relative abundances of Ca and S (soil Cluster 3). The second RDA axis (6.9% variability) opposes bacterial Cluster 4 enriched in Pseudomonadales and soil samples with high relative abundance of P (soil Cluster 2). In contrast to bacterial diversity, the chemical composition of the soil explained 8% of the variance of the putative functions (R^2 adjusted; Figure 4C). P, Fe and Ca were significant (explaining, respectively, 1.3%, 0.9% and 0.9% of total variance; Figure 4D). The

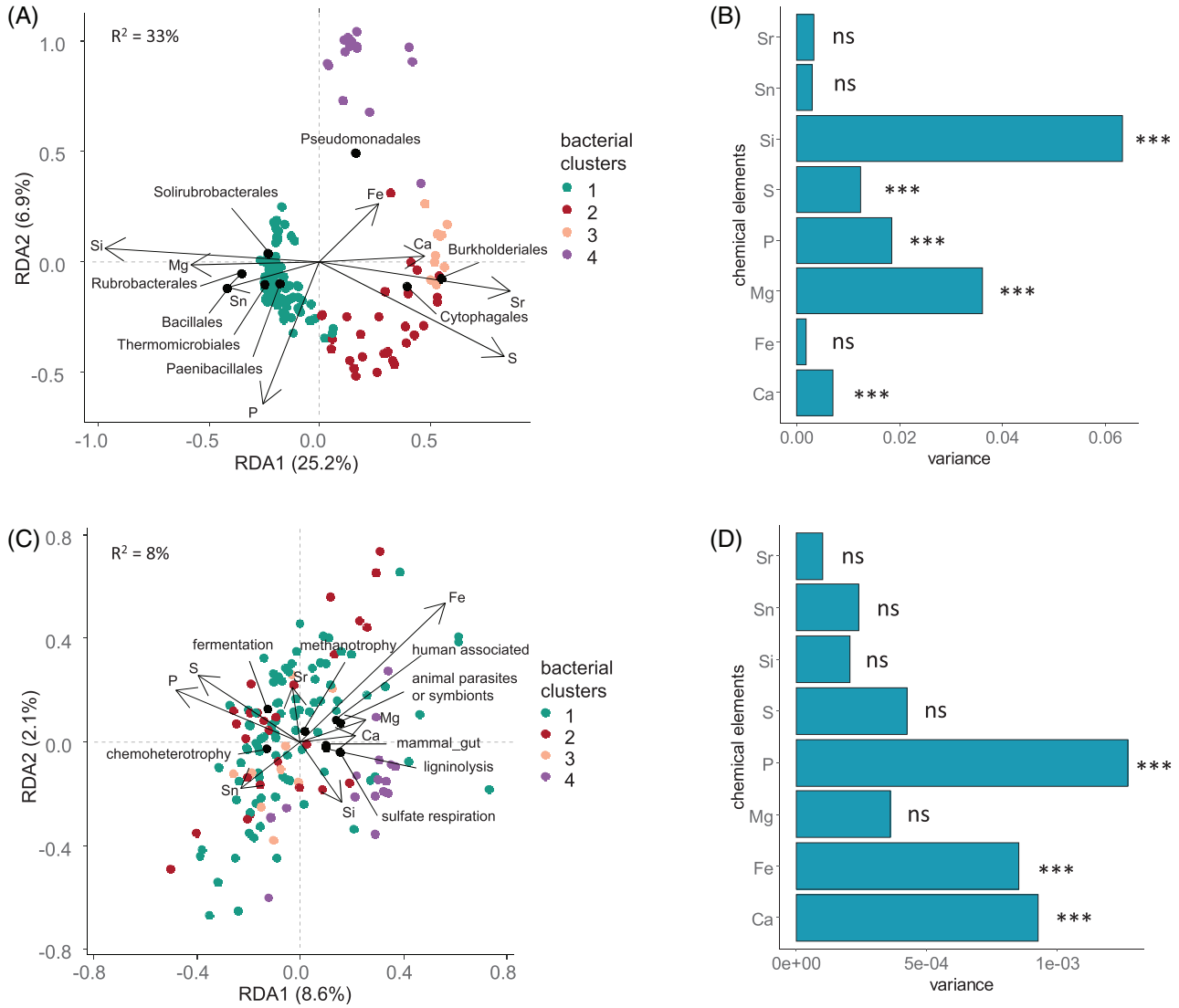

FIGURE 4 Bacterial clusters and functional groups related to the chemical composition of the soil. Plots of the two first axes of the redundant analyses (RDA) of the bacterial diversity at the order level (A) and the functional groups (C) using the eight representative chemical elements of the soil. The corresponding fractions of variance explained by the RDA for each of the eight representative chemical elements of soil, for bacterial abundance (B) and the functional groups (D). Random independent permutation test and Holm correction (p-value < 0.01).

RDA tended to confirm that ligninolysis, sulphate respiration and the human/animal-related functional groups were associated with the presence of the highest Ca content in soils (mainly found inside the Roman fort; soil Clusters 2 and 3), in contrast to the fermentation and the chemoheterotrophy functional groups, which were associated with higher P contents.

DISCUSSION

The Roman fort is a disrupted hot dryland ecosystem

The Roman fort used for this study is located in a hot dryland ecosystem characterised by high temperatures, low but sometimes torrential precipitation, high solar radiation and sparse vegetation (Almazroui et al., 2012). The soils of the natural ecosystems surrounding the Roman fort are sandy (average [Si] = 90%) with very low organic carbon and nitrogen contents (<0.1%; data not shown), which is typical of hot sandy deserts (Makhalanyane et al., 2015). The pH of these soils is higher (pH = 8.0–10.4) than expected for the Arabian Peninsula (pH = 7.0–7.5) (Makhalanyane et al., 2015), which could be related to former volcanic activity in the region. These environmental constraints (high pH, high aridity index and low organic matter) are generally the main drivers of bacterial soil diversity and shape the composition of the bacterial soil clusters in such ecosystems (Delgado-Baquerizo et al., 2018). The bacterial signature found

outside the fort is typical of natural hot dryland ecosystems (i.e., dominance of the Actinobacteria, Proteobacteria, Firmicutes and Chloroflexi phyla), but the Acidobacteria and Planctomycetes phyla, which are usually well represented in high pH soils, were poorly represented in our dataset, suggesting that, in this ecosystem, aridity may have a stronger influence on bacterial diversity than pH (Delgado-Baquerizo et al., 2018; Fierer et al., 2012). The *Firmicutes* phylum is not ubiquitous in dryland ecosystems and its relatively high abundance in the soils surrounding the Roman fort studied here could be explained by the ability of some taxa to form desiccation-resistant endospores and to efficiently colonise the plant rhizosphere in hot arid soils (Prestel et al., 2008; Teixeira et al., 2010). The proximity of the Roman fort was characterised by changes in both the chemical and bacterial compositions of soil and, to a lesser extent, in the functions of the bacterial communities. Analysis of the chemical composition of the soils inside the fort revealed increases in some chemical elements known to be related to past human occupancy (i.e., Ca, P, S, Sr, Cu, Sn, In) (Berger et al., 2019; Williams et al., 2020). These changes in the chemical composition at least partially explain the progressive but marked decrease in bacterial diversity as we approached the Roman fort, resulting in the dominance of two taxa in the soils inside the fort. Overrepresentation (>75%–85%) of one genus or species in an ecosystem is rare, even in extreme environments (Fagorzi et al., 2019; Frindte et al., 2019) and, to our knowledge, is only found in very specific ecological niches such as grape-must fermentation, with the spontaneous dominance of the *Saccharomyces* genus (Guzzon et al., 2022). These unexpected results strongly suggest that the construction of the Roman fort and its human occupancy for four centuries have lastingly modified the soil of this dryland ecosystem, even after 1500 years of human absence. However, differentiating between DNA fragments from living or dead (ancient DNA) organisms in sediments remains difficult, even though methods have been proposed (Carini et al., 2020), and it is consequently not clear if our observations are a direct or indirect picture of the past or the consequences of the past in the present. Furthermore, long-term analyses are needed to assess how long this disturbed ecosystem (i.e., the Roman fort) requires to be fully reintegrated into the surrounding natural environment.

Soils conserved chemical and microbial traces of past human occupancy

The study of the chemical composition of archaeological sediments is not new and proved to be relevant to identify local past human activities in archaeological sites (Pastor et al., 2016; Scott, 2020). The study of the microbial composition of archaeological sediments is more recent and has already enabled the identification of shifts in bacterial communities associated with different sediment layers as well as enrichment in specific human- and animal-associated taxa (Giguet-Covex et al., 2014; Khanaeva et al., 2013; Margesin et al., 2017; Siles et al., 2018). However, these studies were often limited to a few samples of archaeological sediments with no comparison with samples from a distant preserved area. Our study highlights the fact that both the chemical and the bacterial composition of the soil are appropriate tools that are hardly intrusive and can be used at a large scale during the initial surveying of archaeological sites. In particular, they can provide complementary information both to describe the architecture of an archaeological site (e.g., by identifying the location of buried buildings) and by locating past human occupancy, which has locally modified the biotic and/or the abiotic composition of the soil. For instance, we show that changes in the bacterial composition of soils are not clearly linked with changes in the chemical composition; for example, along the exterior transect, where the high relative abundance of an ASV, corresponding to a *Pseudomonas* sp., was only observed on the western part of this transect. In this case, the use of bacterial data made it possible to hypothesise a link between distant areas, whereas the chemical data did not. Interestingly, an operational taxonomic unit (OTU) corresponding to the *Pseudomonas* genus was also found strongly enriched in the two lower layers in the archaeological site of Monte Iato in Italy (Siles et al., 2018). Similar results obtained at two archaeological sites located 2600 km apart suggest that the high relative abundance of this genus in sediments could be a putative marker of past human occupancy. It should nevertheless be noted that DNA barcoding is based on the amplification of small DNA fragments and does not allow the dating of artefacts, consequently, standard archaeological dating methods are complementary to our approach (Schwarcz, 2002).

Chemical and microbial soil compositions reveal additional information about the nature of past events

In addition to enabling the description of an archaeological site, the chemical and bacterial composition of soils can also provide clues about the nature of past events (Williams et al., 2020). For instance, we show that changes in the chemical composition of soils can inform about the putative type of human activities that were performed locally (e.g., the occurrence of fire with enrichment in S, or a putative cooking area with enrichment in Ca, Sr or P). We also show that the databases of functional prediction for microbes, such as

FAPROTAX for bacteria (Louca et al., 2016), can confirm the presence of humans and/or animals in the distant past in an archaeological site (e.g., the increase in human- and animal-associated bacteria inside the Roman fort). However, the functional inference from taxonomic data is still limited by the low taxonomic resolution provided by the short amplified DNA fragments in barcoding methods (Djemiel et al., 2022), but the sequencing of longer fragments and the enrichment of databases will soon allow more accurate predictions (Douglas et al., 2020).

Our work promotes interdisciplinary research and highlights the relevance of combining archaeology and microbial ecology to improve our understanding of past events. We showed that both the abiotic and the biotic compositions of archaeological sediments are highly informative and bring redundant and complementary results with the methods classically used in archaeology. Moreover, the chemical and microbial analyses of archaeological sediments can be used for quickly identifying areas of interest on vast archaeological sites. Finally, the widespread use of abiotic and biotic composition analyses of archaeological sediments will allow us to identify common patterns related to human occupation and will allow us to better predict the lasting consequences of current human actions on natural ecosystems.

AUTHOR CONTRIBUTIONS

Stéphane Boivin: Data curation (equal); formal analysis (lead); investigation (lead); methodology (lead); software (lead); writing – original draft (lead). **Amélia Bourceret:** Data curation (equal). **Kenji Maurice:** Investigation (supporting); writing – review and editing (supporting). **Liam Laurent-Webb:** Investigation (equal); writing – review and editing (equal). **Tomáš Figura:** Investigation (supporting); writing – review and editing (supporting). **Julie Bourillon:** Investigation (supporting). **Jérôme Nespoulous:** Investigation (supporting). **Odile Domergue:** Investigation (supporting). **Clémence Chaintreuil:** Investigation (supporting). **Hassan Boukcim:** Supervision (lead). **Marc-André Selosse:** Supervision (lead). **Zbigniew Fiema:** Methodology (supporting); writing – review and editing (supporting). **Emmanuel Botte:** Methodology (supporting); writing – review and editing (supporting). **Laila Nehme:** Conceptualization (lead); methodology (equal); writing – review and editing (supporting). **Marc Ducousso:** Conceptualization (lead); methodology (equal); supervision (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available in the paper and its Supporting Information files. The raw sequencing data are available at GenBank BioProject PRJNA1037340: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1037340

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SUPPORTING INFORMATION

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