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► **To cite this version:**

Pamela Afouda, Gregory Dubourg, Didier Raoult. Archeomicrobiology applied to environmental samples. *Microbial Pathogenesis*, 2020, 143, pp.104140. 10.1016/j.micpath.2020.104140 . hal-02517885

**HAL Id: hal-02517885**

**<https://hal-amu.archives-ouvertes.fr/hal-02517885>**

Submitted on 22 Aug 2022

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1                    **Archeomicrobiology applied to environmental samples**

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13                    **Body text word count: 114**

14                    **Abstract word count: 6443**

15 **ABSTRACT**

16 The study of ancient microorganisms represents one of the main ways to understand how  
17 microbes have evolved to date, especially those associated with humans or ecosystems of  
18 interest. However, these studies are always tedious because the viability of the microbes is  
19 difficult to maintain and the degradation of their DNA, can make their detection difficult. The  
20 explosion in the number of studies on ancient microorganisms in recent years is partly due to  
21 improved methods and their availability, ranging from microscopy to next generation  
22 sequencing techniques (NGS). In this article, we discuss these methods and their contribution  
23 to deciphering the ancient environmental microbial community, with particular emphasis on  
24 permafrost, ancient halite, amber and ancient rocks.

## 25 INTRODUCTION

26 While the recent dramatic evolution of methods commonly used in the field of microbiology  
27 is mainly used to analyze contemporary specimen, studies on ancient samples have increased  
28 considerably in recent years. Notably, these studies clarify our understanding on the changes  
29 of microbial community at different periods as well as the associated phenomena [1,2],  
30 including climatic conditions [3,4], infectious diseases or outbreaks [5,6]. These studies are  
31 useful for understanding the history of diseases associated with significant human mortality in  
32 the past [7] or discovering the antiquity of certain diseases, long considered to be associated  
33 with modern humans [8]. In addition, studies on ancient samples provide an understanding of  
34 how individuals in microbial communities in an entire population or in an ancient  
35 environment have evolved [9–11]. Among the factors **facilitating** these studies, the reduction  
36 in **the** cost of high throughput sequencing techniques, as well as the advanced and advent of  
37 other OMICS strategies have **contributed widely** to the field [2,5,12].  
38 In this review, we **propose first** to detail the methods used to recover ancient microorganisms  
39 through several examples and then to highlight the conclusions of environmental studies in  
40 terms of ancient microbes' diversity, through examples such as permafrost, ancient halite,  
41 amber and ancient rocks that **have prompted** several investigations.

42

## 43 BIBLIOGRAPHICAL METHODS

44 A literature was manually performed through Google, PubMed, Web of Science and Google  
45 Scholar databases covering the period from September 1928 to August 2019 using the  
46 following keywords: “ancient microbes”, “ancient microbiome”, “ancient bacteria evolution”,  
47 “paleomicrobiology”, “permafrost”, “ancient rocks”, “ancient halite”, “amber”, “prehistory of  
48 antibiotic resistance”, “resistance mechanisms in ancient microorganism”, “resistance  
49 mechanisms in permafrost”, “contaminant in ancient environment sample”, “contaminant in

50 permafrost sample”, “contaminant in amber sample”, “contaminant in ancient rocks sample”,  
51 “contaminant in ancient halite sample”.

52 In view of the many investigations we found using the keyword "permafrost", we also  
53 performed a more specific literature for "Permafrost" by performing the queries specific to  
54 each database (Table 1).

55 Literature allowed us to identify 51512 scientific articles, among which we selected 343  
56 articles based on of their summaries and titles. Finally, 172 articles were retained for this  
57 review given their relevance for this work (Figure 1).

58

## 59 **TECHNIQUES USED FOR THE DETECTION OF ANCIENT MICROORGANISMS**

60 Techniques for studying ancient microorganisms use **multiomics** techniques including  
61 microscopy techniques, culture and DNA-based approaches.

### 62 **Microscopy**

63 Microscopy allows an open approach for screening bacteria, fungi, parasites or other  
64 microorganisms in ancient samples and highlights their different forms of survival in ancient  
65 environments. In ancient human samples, different parasites (such as helminths) and their  
66 eggs along with cysts and molds can be easily detected by light microscopy in coprolites and  
67 rectum of a mummy dated middle age or even before [13–15]. Transmission **Electron**  
68 Microscopy (TEM) was also used in ancient samples studies, and its superior resolution to  
69 light microscopy makes it possible to identify calcified and non-calcified bacterial species in  
70 archaeological studies [16]. TEM technique was used to study long-term survival mechanisms  
71 of bacteria in permafrost and their physiology [17,18]. Indeed, using TEM technique, the  
72 shapes of the intact bacterial species found were almost identical regardless of the age of the  
73 permafrost. These small cells housed capsular layers, thickened cell walls and a non-  
74 homogenous or/ and dense cytoplasm that would allow bacteria to survive in this extreme

75 environment [17,18]. Used as a prelude to Omics techniques, the electron microscopy also  
76 makes it possible to identify new bacterial taxa in permafrost [19]. Also, both bacilli and cocci  
77 were revealed in fluid inclusions in ancient halite from different depths by TEM [20,21].  
78 Another microscopy technique, scanning electron microscope (SEM), has been used to detect  
79 cribriform and rounded bacteria, but also bacterial molds from ancient samples [22] and  
80 revealed the microbial morphology and the formation of biofilm in permafrost samples [17].  
81 After staining with DAPI, SEM has also been associated with light microscopy to identify and  
82 categorize different morphologies of microbial cells from permafrost samples [23]. In  
83 addition, the use of epifluorescence microscopy has been reported and has revealed the  
84 presence of prokaryotic and eukaryotic microorganisms on the surface of ancient seeds buried  
85 for 30,000 years in permanently frozen permafrost [24]. Fluorescence microscopy coupled  
86 with three other techniques: Live / Dead staining, endospore enrichment, and selective  
87 depletion of dead cell DNA provide a means of differentiating dormant, dead, or active  
88 microbial cells in ancient permafrost samples [25]. This differentiation has made it possible to  
89 highlight in permafrost samples aged 19,000, 27,000 and 33,000 years, that dormancy is also  
90 part of one of the strategies used by microorganisms to survive long-term in permafrost [25].  
91 This finding shows that although microbes have the ability to sporulate, some (*Clostridia*)  
92 prioritize survival in their vegetative forms in this extreme environment [25].

### 93 **Culture techniques**

94 Culture techniques have greatly contributed to the understanding of the biodiversity and  
95 microbial community associated with ancient samples. These techniques imply that  
96 microorganisms are still alive in dormant or active state in the samples before studies  
97 [11,21,26,27]. Microorganisms dormant in ancient samples such as permafrost can be  
98 activated after samples treatment with chitin [28]. These culture techniques provide microbial  
99 isolates, whose identification is generally performed by 16S rRNA gene sequencing [29].

100 Culture media reported in the literature are varied, from oligotrophic media to nutrient-rich  
101 media. **However**, in most cases, these are oligotrophic culture media that can isolate the most  
102 microorganisms from ancient environmental samples after long incubation period (up to 15  
103 weeks or more) [30]. Usually, the culture media reported for the culture of the ancient  
104 permafrost samples are nutrient-poor media, such as the R2A (Reasoner's 2A) [29,31] or PYG  
105 (Peptone, Yeast Extract, Glucose) [31] medium. **Yet**, nutrient media, such as LB (Luria  
106 Bertani), TSA (trypticase soy agar) have been used for the **culture** of Siberian and Canadian  
107 permafrost microorganisms [26,32]. These media can be enriched with vitamins or made  
108 selective by the addition of antibiotics [26,32] or by the addition of nitrite [19] to optimize the  
109 culture of microorganisms as reported for the culture of amber samples [33–35] as well as **the**  
110 culture of permafrost bacteria oxidizing nitrite [19]. Oriented media, such as halophilic media,  
111 with different salt concentrations have also been reported, particularly in permafrost [36], but  
112 also in halite samples [21]. These culture media included Casamino Acids Binghamton (CAB)  
113 media, Pyruvate Glycerol Binghamton media [21].

#### 114 **Molecular diagnostic techniques**

115 The first sequencing of an ancient DNA dates back to 1984 and focused on the study of DNA  
116 from extinct ancient species, including the sequencing of mitochondrial DNA from a museum  
117 specimen of the zebra species quagga (*Equus quagga*) [37]. Since then, curiosity for the  
118 molecular evolution, behavior and ecology of ancient DNA has increased and the techniques  
119 being used now combine PCR, cloning and standard sequencing [38] on the one hand, but  
120 also next generation sequencing techniques (NGS) [2,38,39], metagenomics [40–42] and  
121 microbial detection arrays [43] on the other. DNA-based approaches are nowadays the most  
122 widely used in the study of ancient samples because they provide a better understanding of the  
123 origin and evolution of ancient species than culture techniques [44]. The use of NGS methods  
124 for the study of ancient DNA microorganisms is expanding [40,42,45], but other techniques,

125 such as mass spectrometry techniques [40,46–49], fluorescent in situ hybridization techniques  
126 [50] and immunoassay techniques [51–54], are also widely associated with ancient  
127 microorganism studies.

### 128 *DNA extraction*

129 DNA extraction is one of the most delicate parts for all studies based on ancient DNAs. In  
130 fact, not only the ancient DNA is confronted with multiple contaminants including inhibitors  
131 of PCR, but especially with numerous damages thus generating molecules of small sizes [55].  
132 Ancient DNA faces oxidative damage, which significantly affects cytosine and thymine bases  
133 [55]. Authors suggest treatments of its ancient DNA extracts with alkali and various enzymes,  
134 such as endonuclease IV; endonuclease III; uracil-DNA glycosylase + endonuclease IV [55].  
135 More specifically with regard to DNA extracts from ancient sediments, some authors  
136 described procedures for the appropriate extraction of environmental DNAs (contained in  
137 frozen sediment cores) **with appropriate solutions to remove** PCR inhibitors [56]. This  
138 protocol includes pre-treatment of the sample under rigorous aseptic conditions; and essential  
139 stages of total sediment dissolution allowing optimization of the amount of DNA [56].  
140 The extraction of genomic DNA from permafrost samples for example, use commercial kits  
141 suitable for extracting DNAs present in the environment, water or soil among which the  
142 highest gDNA concentrations noted **come** from the FastDNA SPIN Kit for Soil (MP  
143 Biomedicals, Irvine, CA) kit [57].

### 144 *Polymerase chain reaction (PCR) and its variants*

145 The objective of the PCR technique is to obtain several copies of a targeted DNA, initially in  
146 small quantities in the samples to be analyzed, so it was included very early in the study of  
147 ancient DNA. This has greatly facilitated the diversification and remarkable perspective that  
148 we now have on ancient DNAs [55,58]. Different types of PCR are performed on ancient



149 DNAs. These PCRs include standard PCR, quantitative PCR, multiplex PCR and nested PCR,  
150 suicide PCR and immuno-PCR.

151 ✓ *Standard PCR*

152 Since there are several degradation conditions specific to ancient samples, including damaged  
153 bases, weak DNA fragments that can be degraded and the presence of PCR inhibitors  
154 associated with ancient DNA, modifications have been suggested in the commonly used  
155 standard PCR protocol [59,60]. **Therefore**, it is recommended to carry out several PCRs on  
156 different extracts of the same sample to compare the results of the PCRs [60,61]. Also, these  
157 modifications include pretreatment with bovine serum albumin (BSA) [62], with uracil DNA  
158 glycosylase (UNG or UDG) [63], or N -phenacylthiazolium bromide (PTB) [64], but also  
159 isopropanol precipitation [59] to remove PCR inhibitors co-extracted with DNAs. Standard  
160 PCR systems used to amplify ancient microbial DNAs sometimes have different targets. The  
161 16S RNA **Gene Region** is usually the most targeted in the study of haloarchaeal diversity  
162 [65,66], but also bacterial diversity [54]. The ITS region is often the one targeted in the fungal  
163 community study [66], but also the algal community [67]. As for the study of eukaryotes, **18S**  
164 **rRNA gene**, cytochromes or NADH dehydrogenase can be targeted [54].

165 ✓ *Multiplex PCR and Quantitative PCR (qPCR)*

166 The advantage of multiplexed PCR in the analysis of ancient samples lies in its ability to  
167 have several amplified fragments simultaneously in a single PCR [68]. This would allow,  
168 even from very small quantities of ancient samples, to have enough easily analysable DNA  
169 fragments [68]. Based on what already existed, Stiller and Fulton proposed a multiplex PCR  
170 protocol better suited to target the ancient highly degraded microbial DNAs [68]. This  
171 protocol includes two steps: a first step that consists of a normal multiplex PCR and a  
172 second step that uses this first multiplex PCR product as matrix, along with adding nested  
173 primers that increase the specificity and selectivity of the PCR reaction [68]. As for the

174 qPCR, it has also been reported in the study of ancient samples. Used on permafrost, it has  
175 recovered up to  $37.5 \pm 1.9$  % total genomic DNAs [57]

176 ✓ *Nested PCR*

177 Much more sensitive than conventional PCR, nested PCR has the advantage of a high level  
178 of amplification of the target DNA. It consists in amplifying the PCR products resulting  
179 from a first reaction of the conventional PCR [68]. The involvement of nested PCR in  
180 determining the microbial community of the ancient samples has been much reported  
181 [61,68]. Associated with culture, this technique has proven effective in comparing the  
182 archaic community at different depths of ancient halite samples dated to the Pliocene (5.3 to  
183 1.8 million years) [61].

184 ✓ *Suicide PCR*

185 “The suicide PCR” has been designed to overcome the problems of false positives in PCR  
186 techniques usually caused by cross-contamination. Indeed, the principle of the “suicide PCR”  
187 relies in a unique utilization of the same PCR primers without a positive control in the same  
188 laboratory [5]. This protocol confirmed the presence of *Yersinia pestis* DNA in dental pulp  
189 from skeletons of Black Death victims [5,69]. Moreover, this technique allowed the  
190 identification of *Bartonella quintana* in 4,000-year-old human remains [70]. Preceded by a  
191 **rapid strip test**, it also proved effective for the detection of *Yersinia pestis* antigen F1 in  
192 human remains, corresponding to the victims of the plague of the **16<sup>th</sup>, 17<sup>th</sup> and 18<sup>th</sup> centuries**  
193 **in the south-east of France** [6].

194 ✓ *Immuno-PCR*

195 Immuno-PCR is a technique that has been developed in 1992, which combines the advantages  
196 of immunodetection and the strong amplification and specificity of PCR, thus forming a very  
197 strong antibody-DNA complex capable of detecting very low amount of microbial DNAs  
198 [71]. This technique allows the amplification of DNA sequences from ancient samples, which

199 are sometimes difficult to obtain with a simple standard PCR amplification (Sano, Smith, and  
200 Cantor 1992). So far, its use has only been reported once in the microbial detection of ancient  
201 samples [72]. It exhibited a higher sensitivity compared to conventional ELISA and PCR  
202 techniques for the detection of plague agent *Yersinia pestis* in the dental pulp samples taken  
203 from Black Death victims [72].

#### 204 ***Molecular cloning techniques***

205 Ancients DNAs often undergo damage by oxidative processes [55], by hydrolysis processes,  
206 by DNA crosslinks or also degradation by microorganisms' nucleases in the post mortem cell  
207 [38] that can affect their matrix [73], and this does not guarantee the authenticity of results  
208 from sequencing. For this, the cloning of independent amplicons is privileged by some  
209 researchers as one of the major precautions before sequencing in order to minimize the  
210 determination of incorrect sequences of ancients DNAs [73,74].

#### 211 ***Next generation sequencing (NGS) techniques***

212 Next-generation sequencing provides an easy way to study the microbial community in  
213 ancient samples [2] by its rapidity to sequence the microbial genomes. The sequencing  
214 strategies for ancient samples studies included Illumina MiSeq sequencing strategy [45],  
215 Roche 454 GS FLX Titanium pyrosequencer [75]. Their use has facilitated advances in  
216 microbial genomic studies, particularly on the history of certain infectious diseases, but also  
217 on the bacterial ecology [76]. For instance, the genomic reconstruction of *Clostridium*  
218 *perfringens* found in the soft tissue of frozen mummified sample (Tyrolean Iceman) revealed  
219 virulence genes, which would specialized very early in these bacteria, while in the  
220 *Pseudomonas veronii*, these virulence genes would be acquired for its adaptation to its  
221 ecological niche [41]. Applied to old calcified plaque, it reveals that the presence of chronic  
222 diseases in the subjects of the Industrial Revolution is due to a low diversification of  
223 microorganisms compared to the Neolithic oral microbiota [2]. However, when applied to

224 excrement found in archaeological contexts, a change in its microbial community was  
225 observed, compared to the modern community [75].

### 226 *Shotgun sequencing (metagenomics)*

227 The advent of metagenomics makes it possible to characterize the microbial community of the  
228 16S rRNA gene in a sample much more quickly, making it possible to characterize complex  
229 communities in the environment [77,78]. In contrast to culture, metagenomics provides a  
230 much faster overview of the microbial community, and allows comparative genomic studies  
231 [79]. Metagenomics contributes to the identification and taxonomy of ancient bacterial  
232 species [77] and provides a better understanding of the microbial life [80], the microbial  
233 diversity and the metabolic functions [78] under extreme conditions and the impact of age on  
234 the microbial community in permafrost samples several million years old.

### 235 *Fluorescent in situ hybridization (FISH)*

236 Fluorescence in situ Hybridization (FISH) technique reveals the different morphologies of  
237 cultivable and non-cultivable colonies contained in a sample. This molecular technique uses  
238 labeled fluorescent probes to detect nucleic acid sequences contained in the cell itself. These  
239 targets include RNA or DNA at different chromosomal phases of the cell [81], thus the  
240 metabolic activities of cells greatly influence FISH results [82]. Its high efficiency for the  
241 rapid detection of pathogenic microorganisms has been widely reported, to such an extent that  
242 it is recommended as one of the routine diagnostic tools [83–85]. This technique is also very  
243 effective for reliable quantification of various microbial taxa involved in anammox processes  
244 into complex environments such as the black sea [86]. Its effectiveness is real in assessing the  
245 microbial community and its use on the soil of the Siberian tundra has shown that a decrease  
246 in the microbial cell community is more significant in the deep layers [50]. FISH was also  
247 effective in evaluating the number of bacterial cell and archaea metabolically active in

248 permafrost samples after exposure to a 100 kGy irradiation dose, thus revealing that  
249 microorganisms in this environment are radiation-resistant [31].

### 250 ***Microbial Detection Array***

251 Given the degradations often observed on ancient samples, studies focusing on microbial  
252 pathogens based on ancient DNA sequence analysis are sometimes very expensive [43].  
253 Microarray technologies, have recently been shown to be effective in detecting  
254 microorganisms in ancient samples [43], as they are very fast and less expensive than  
255 sequencing techniques [87]. Several types of microarray systems have been reported for the  
256 study of ancient samples. **The most reported microarray system is based on the use of long  
257 oligonucleotide probes (50-65 bp) and constitutes a database named Lawrence Livermore  
258 Microbial Detection Array (LLMDA) containing at least a total of 388,000 oligonucleotide of  
259 microorganisms [12,87]. This enables to identify simultaneously and very quickly the  
260 genomic content of several microorganisms contained in a sample [87]. For example, the  
261 LLMDA technique has allowed the rapid identification of *Vibrio cholerae* and *Yersinia pestis*  
262 strains in tooth samples from 19th century and a medieval period, while their sequences  
263 recovered only weakly with high-throughput sequencing techniques [43].** Otherwise, Yergeau  
264 *et al.* have extensively investigated the use of much more specific microarray for the  
265 screening of 16S rRNA genes of polar soil microorganisms [77,88,89]. The most widely used  
266 microarray database for cold environments such as permafrost [77] consists of 525 25-mer  
267 oligonucleotides targeting several bacterial and archaeal taxa [77,88].

### 268 **Immunoassay techniques**

269 Numerous studies have demonstrated the sensitivity and specificity of immunoassay  
270 techniques for the detection of parasitic coproantigens in contemporary samples [90–94].  
271 Although the elements contained in the ancient samples may be damaged by time due to  
272 adverse conditions, immunoassay techniques have, in several cases, supplemented

273 microscopic analysis by allowing the detection of parasites in ancient samples [52]. ELISA is  
274 an immunoassay technique, easy to perform, which consists of a monoclonal antibody assay  
275 kit. An antibody-antigen complex will therefore be formed with the antigen of the  
276 microorganisms and the reaction is revealed by a color indicator due to the enzyme fixed on  
277 the antibody [52]. For example, the detection of ancient intestinal parasites' coproantigen,  
278 such as *Giardia duodenalis* coproantigen [52] and *Entamoeba histolytica* coproantigen [53]  
279 has been easily achieved through the use of enzyme-linked immunosorbent assay (ELISA).  
280 In addition, the detection of microbial antigens permafrost was made possible by an  
281 immunosensor named LDChip300 (Life Detector Chip) with hundreds of antibodies. This  
282 technique allowed the determination of several bacterial clusters (*Firmicutes*, *Proteobacteria*,  
283 *Bacteroides*, *Actinobacteria*) and archaeal clusters (mainly *Methanobacterium* spp) [23]

#### 284 **Mass spectrometry**

##### 285 ***Matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry*** 286 ***(MALDI-TOF MS)***

287 Matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry  
288 (MALDI-TOF MS), based on the analysis of the proteins, has proven in recent years to be a  
289 fast and efficient tool for the detection and classification of proteins from ancient samples  
290 [47–49,95,96]. **As for the determination of microorganisms**, its use in the analysis and  
291 identification of 1400-year-old mycolic acids, a tuberculosis biomarkers [97], has opened a  
292 way on **for** its possible efficacy in the study of ancient microorganisms. Recently used as  
293 screening for bacterial identification in a culturomic study, it allowed us to **obtain** a unique  
294 protein profile of a new bacterial species of the genus *Bacillus* isolated in Siberian permafrost  
295 [98]; whose species-level identification and complete characterization required the sequencing  
296 of the 16S rRNA gene [99].

##### 297 ***Metaproteomic***

298 Metaproteomic is a method of analysis of non-nucleoside biomolecules. Associated with  
299 metagenomics for microorganisms study, it provides all the proteomic information contained  
300 in a complex sample [46]. This technique uses tandem mass spectrometry (MS/MS) to  
301 generate protein profile, peptide signals, whose correspondences is determined by the  
302 available bioinformatics resources such as the Byonic™ database [100]. Using the  
303 metaproteomic approach on 120,000-year-old bone samples from the Ziegler Reservoir fossil  
304 site in Snowmass Village, Colorado, the authors reported the presence of “Hydroxylysine  
305 glucosylgalactosylation”, a post-translational modification of collagen, which would be  
306 strongly involved in the conservation of organic matter, as well as new peptide and protein  
307 sequences, whether or not derived from the extracellular matrix [100]. For instance, the study  
308 of microorganisms in medieval dental calculus by metaproteomic revealed well-preserved  
309 proteins with abundant and clinically important proportions, such as proteins of periodontal  
310 pathogens: *Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola* [101].

### 311 ***Raman spectroscopy***

312 Raman spectroscopy, also known as Raman spectrometry, is involved in the study of ancient  
313 microorganisms as a non-destructive method of searching for microbial biomarkers [102]. Its  
314 sole use in paleomicrobiology has been reported in the study of ancient halite microorganisms  
315 and consists in identifying, in a record time, biomolecules belonging to microorganisms  
316 integrated into fluid halite inclusions. The entrapped amino acids detected included glycine,  
317 L-alanine,  $\beta$ -alanine, L-serine and  $\gamma$ -aminobutyric acid [103]. Recognition of the  
318 microorganism is made possible by the interpretation of spectra generated by biomarkers  
319 [102–104].

### 320 **Contaminants**

321 Microbial diversity is important in environmental samples, but one of the major problems that  
322 often raises doubts about their authenticity is the contamination of ancient samples with

323 external material [105,106]. The contaminants involved may be other exogenous  
324 microorganisms or viruses contracted due to carelessness in sampling or laboratory handling  
325 [106] or reagents used in handling [107]. Moreover, **it is sometimes** difficult to avoid  
326 contamination despite precautions. For example, in constantly cold environments, ice tissue  
327 cracks or thawing are also a constant source of contamination [108,109] because they increase  
328 hydraulic conductivity and allow solute movement that promotes the migration of dissolved  
329 or insoluble contaminants [109]. Consequently, a heterotrophic microbial population emerges  
330 greatly at contaminated thawed sites compared to uncontaminated sites [108].

### 331 **Contaminants management**

332 To avoid the contaminants caused by a lack of standard precautions, the authors suggested  
333 strict asepsis and antiseptic conditions consisting mainly of the use of sterile disposable  
334 equipment (wearing gloves, masks, caps, etc.), the use of a clean laboratory dedicated solely  
335 to the handling of ancient samples, systematic cleaning of all PCR equipment before handling  
336 [106]. **Then, decontamination techniques on ancient samples such as permafrost , amber,**  
337 **ancient halite and ancient rocks samples have been proposed** [61,67,110–112] **to avoid any**  
338 **amplification of DNA from contaminants.** The various decontamination protocols included:  
339 the use of ethanol and/or a Bunsen lamp; use of acid; use of bleach wash; use of combination  
340 of 6% (w/v) sodium hypochlorite saturated NaCl, NaOH, saturated NaCl, HCl, saturated  
341 Na<sub>2</sub>CO<sub>3</sub>/NaCl; use of saturated NaCl and 70% (v/v) ethanol [61,112]; use of peroxide,  
342 ethanol and heat [113,114]; use of 2% Glutaraldehyde [115].

343

### 344 **APPLICATIONS TO DIFFERENT ENVIRONMENTAL MICROBIOMES**

345 The environmental microbiome is filled with all types of microorganisms and certainly the  
346 oldest known microorganisms on earth [106]. Regarding environmental microbiome studies,  
347 the case of permafrost has been the subject of extensive studies in recent decades



348 [11,116,117] because this environment is constantly at a low temperature and capable of  
349 containing and preserving ancient DNAs for thousands of years [106]. Its microorganisms  
350 adapt to their environmental conditions and, **as a result**, their survival modes vary according  
351 to the environment in which they are found. In samples collected in cold environments for  
352 example, the GC content and sporulation of the isolates can be a mean of survival [32].  
353 Indeed, about 30% of the isolates found in permafrost are sporulated [32]. In general, the GC  
354 contents of 16S rRNA gene sequences from permafrost are related to their optimum growth  
355 temperature. In fact, a high GC content is related to a high growth temperature and vice versa.  
356 In permafrost, the GC content can reach 72-80% with an optimal growth temperature that can  
357 also reach 55-80°C [118]. GC high content isolates are linked to samples several million years  
358 old, while low-content GC isolates are linked to many recent samples only a few thousand  
359 years old [32].

### 360 **Permafrost**

361 Permafrost is defined as a soil that has been frozen for at least two years. It currently **accounts**  
362 **for almost** 25% of the **Earth's** surface and is mainly found in Alaska [27], Siberia  
363 [18,116,119], **the** Antarctic [120] and Canada [26,36,121], but also in ice-free regions, such as  
364 the alpine mountains of Europe [122], America and China [123]. Despite extreme conditions,  
365 many endogenous culturable microorganisms have been reported, and the mesophilic,  
366 psychotropic, halotolerant, but also acidotolerant species are represented [121]. In general,  
367 culture-independent methods, such as sequencing identification of the 16S rRNA gene, are  
368 combined with culture-dependent methods to determine microbial diversity of permafrost.

### 369 **Microbial diversity**

370 The cultivable bacteria in permafrost soil are represented by the 4 major phyla : *Firmicutes*,  
371 *Actinobacteria* (with a predominance of order *Actinomycetales*), *Proteobacteria*, and  
372 *Bacteroidetes* [26,116,117,119,124,125]. *Bacteroides* are predominant in high pH soils,

373 whereas *Proteobacteria* are associated with low pH and high nitrogen and carbon levels  
374 [117]. In addition, the implication of genes belonging to *Actinobacteria* and *Bacteroidetes* on  
375 the degradation processes of the Earth's organic carbon stored in Arctic permafrost peatlands  
376 has recently been reported [126]. The advance of molecular high throughput sequencing  
377 techniques revealed a different picture as *Acidobacteria*, *Gemmatimonadetes*, *Actinobacteria*  
378 and *Proteobacteria* [121,127] are found in high proportion from both in the active layer and  
379 in the permafrost. The cultivable bacteria genera found in the permafrost soils are diversified.  
380 The genus *Arthrobacter*, *Planococcus* and *Bacillus* are the most commonly isolated in all  
381 types of permafrost, probably because they are easier to grow and require less nutrients  
382 growth than others [26,36,99,116,117,119,121,124,128] (Table 2). Table 2 summarizes the  
383 most isolated bacterial genera by culture in different types of permafrost. Some of these  
384 bacterial species isolated from permafrost samples may exhibit antimicrobial activities against  
385 pathogenic microorganisms. For instance, *Bacillus tequilensis* and *Pseudomonas spp* isolated  
386 from Canadian permafrost, have demonstrated inhibitory activities against foodborne  
387 pathogens such as *S. aureus*, *L. monocytogenes*, *S. enterica* and *E. coli* O157:H7 [29].  
388 Otherwise, the probiotic activity of *Bacillus cereus* isolated from permafrost on *Salmonella*  
389 has also been reported [129].

390 The 16S sequences of the majority of these bacterial isolates are extremely close, sometimes  
391 identical to those reported in various environmental or human samples [121]. More precisely,  
392 apart from a few genera (Table 2), all others are associated with humans [130].

393 In addition, the microbial diversities obtained in the permafrost soil and in the active layer  
394 depend on the techniques used and the origin of the permafrost [121,122,131,132]. For  
395 example, the most abundant bacterial diversity was identified in the Canadian permafrost soil,  
396 even if the number of viable aerobic cells found in this soil can be up to 100 times lower than  
397 that of the active layer [121]. However, other studies have shown that the greatest microbial

398 diversity of permafrost (Siberian, Greenland ) can be found in the active layer [131,132].

399 Sediments of permafrost are also often rich in methanotrophic bacteria [133], highlighting that  
400 methane can be used by bacteria living there as a source of carbon and energy for their  
401 survival in such an extreme environment. Furthermore, the microbial diversification of  
402 permafrost also widens to molds [27,120] and yeasts [27,134], whose determination is based  
403 on the analysis of internal transcribed spacer (ITS2) sequences in total DNA of sample [120],  
404 but also 18S rDNA sequences analysis [134]. The yeasts isolated in the permafrost are mainly  
405 represented by the genera *Mrakia* and *Leucosporidium* [27], known to adapt to cold  
406 environments [134–136].

407 With regard to molds, a wide variety of cosmopolitan fungal species have been found in  
408 different types of permafrost [27,120,123], of which more than 26 are viable in the Antarctic  
409 permafrost [120]. Among them, a predominance of *Ascomycota* in Chinese and Antarctic  
410 permafrost [120,123] and *Geomyces* in Alaskan permafrost [27] has been reported. Besides,  
411 genera *Penicillium* and *Cladosporium*, known for their spores, adaptations and resistance to  
412 climate change, have been isolated in the Antarctic permafrost [120].

413 In addition to bacteria, archaea are also present in permafrost through DNA-based approaches  
414 [121,137]. Archaea can reach 0.1% of the total 16S rRNA gene copy number of permafrost  
415 [121]. Analysis of DNA fragments from 5 permafrost samples collected from a borehole in  
416 the Kolyma River plain (northeastern Russia) revealed a high diversity of archaea at a depth  
417 of more than 20 meters [137]. The *Euryarchaeota* (especially methanogenic archaea) were  
418 found abundant in both Russian and Canadian permafrost [26,137]. The amount of methane  
419 released in permafrost can be significant, especially just after a period of thawing permafrost  
420 [80]; this gas can be a source of energy and carbon for the anaerobic oxidation of microbial  
421 metabolism, which also explains the important quantity of OTU related to methanogens and  
422 methanogenesis genes found in different types of permafrost [36,80]. The abundance

423 functional metabolic genes present in the permafrost vary with the age and freezing state of  
424 the permafrost, which influences the variation of its microbial community [80]. To survive,  
425 the microbial community develops adaptive traits to acquire new genes and beneficial  
426 pathways depending on permafrost conditions [80]. **Therefore**, compared to younger  
427 permafrost sample, older permafrost sample is associated with enrichment of certain Kyoto  
428 Encyclopedia of Genes and Genomes (KEGG) pathways [80]. A thaw of permafrost can lead  
429 to a decrease in nitrogen fixation genes and an increase in those of denitrification [138].  
430 Besides, the age of the permafrost is correlated with the abundance of aromatic hydrocarbon  
431 degradation genes [80]. The acquisition of adaptive traits for survival in hostile environments  
432 of permafrost is facilitated by several elements, including horizontal gene transfers [139],  
433 chemotaxis, the synthesis of stress-related genes, including those involved in the synthesis of  
434 polyamines [80], but also metabolic activity of some sporulated bacterial species of  
435 permafrost [140]. The increase in other KEGG pathways involved in cell envelope  
436 components, amino-acid and peptide metabolism, carbohydrate metabolism, environmental  
437 sensing and response, membrane transport and the degradation of recalcitrant biomass have  
438 also been reported in ancient permafrost [140].

#### 439 **Other environmental microbiomes**

##### 440 *Ancient rocks*

441 Several studies have **already** reported the presence of microorganisms in rocks dating from the  
442 Precambrian period (ie the period between 4.55 billion years and 540 million years).  
443 **Nonetheless**, very little information is available regarding the culture of microorganisms from  
444 ancient rocks. The scientist Chas. B. Lipman, first reported the presence of living bacteria  
445 living in ancient rocks, and especially in a Pre-Cambrian rock from the Algonkian in Canada  
446 [114]. In particular, rod shaped and sporulated bacteria represented the groups of bacteria  
447 found there [113]. In addition, using scanning microscopy, various microorganisms

448 (Cyanobacteria and other bacteria) have been identified in rocks of Karelia dating back to the  
449 archaean period. These microorganisms are mostly cocci-shaped, sometimes in division.  
450 Some of these, arose in the form of Archean fossils (suspicion of *Melanocyrrillium*) and others  
451 in enthalic fossils [141]. Later, analysis of the microbial composition of ancient rocks  
452 collected in the Yungang Grottoes by combining PCR techniques with denaturing gradient gel  
453 electrophoresis (DGGE) and cloning sequencing showed the predominance of phyla of  
454 *Gammaproteobacteria*, *Firmicutes* and *Alphaproteobacteria* , but also *Sphingobacteria*, and  
455 *Actinobacteria* by phylogenetic analysis [142].

#### 456 ***Ancient halite***

457 Halite or rock salt (NaCl) is a crystalline formed of salts resulting from dried saline  
458 environments [143]. Despite its adverse conditions, many microbiological and geological  
459 studies have revealed the survival of microorganisms in fluid inclusions of ancient halite. The  
460 techniques used included microscopy, Raman spectroscopy, culture and also metagenomics  
461 studies [20,21,61,67,102,144–146]. Although halite may contain ancient microorganisms, the  
462 community diversity remains low. There are very little cultivable bacteria, and cultured  
463 archaea were mostly halophilic. Several ribosomal 16S sequences found in the halite are  
464 attributed to known microbial species but also to new species [110]. In the halite, the species  
465 of long-term survival are mainly halophilic species and more specifically *Halobacterium*  
466 species. Indeed, halophilic and halotolerant oriented culture media have made it possible to  
467 obtain in halite dating back thousands and millions of years, an archaeal community  
468 predominated by the isolates *Haloarcula*, *Halobacterium*, *Halococcus*, *Halolamina*,  
469 *Halorubrum*, *Haloterrigena*, *Natronobacterium*, *Natronomonas* [21,61,110]. Besides, the  
470 bacterial community includes the genus *Oceanobacillus* and *Virgibacillus* isolated from 34–  
471 49 million years old halite (Yunying mine, China ) [147] and from 250 million years of age  
472 halite (Salado formation, USA) [146], respectively. The ribosomal 16S sequences in halite

473 samples represented in abundance the genera *Acinetobacter*, *Burkholderia* and *Pseudomonas*  
474 in halite dating 65–96 million years from Khorat plateau, Thailand, while *Stenotrophomonas*  
475 genus was found in halite dating 11–16 million years ago from Wieliczka mine, Poland [143].  
476 The archaeal 16S rRNA sequences were composed of *Haloarcula*, *Halobacterium*,  
477 *Halobiforma*, *Halomicrobium*, *Halonotius*, *Halorhabdus*, *Halorubrum*, *Halosimplex*,  
478 *Natronomonas* from halite dated of thousands or millions of years  
479 [61,65,110,112,143,148,149]. The long-term survival of *halobacteria* would be enhanced by  
480 their trapping in salt crystals over time [150].

#### 481 ***Amber***

482 Amber is a fossil resin that has the natural **property** of preserving microbial cells and their  
483 DNAs, **making** this sample a good candidate for evolutionary microbiological studies [151].  
484 Several studies have already reported the presence of microorganisms in amber [33–  
485 35,151,152]. The methods for identifying **the** microorganisms reported include comparison of  
486 FAMES, enzymatic profiles, culture and 16S rRNA sequencing [33,34,115]. *Bacillus*  
487 (sporulated species) and *Staphylococcus* genera are predominant in the majority of ancient  
488 ambers of all ages [34,66,115]. Moreover, Dominican amber preserved 25- to 40-million-  
489 year-old was characterized by *Curtobacterium*, *Paenibacillus*, and *Brevundimonas* genera  
490 isolates [33,34,115,152]. The genera *Micrococcus*, *Rathayibacter*, *Nocardioides*,  
491 *Caryophanon*, *Arthrobacter*, *Streptomyces*, *Amycolatopsis*, *Nocardioides*, *Micromonospora*  
492 were also predominated in Dominican amber and Israeli amber preserved about 120 million-  
493 year-old [34,35]. These bacteria are mostly bacterial genera in the soil and environment, but  
494 some are also the main producers of antimicrobials [153–155]. The long-term survival of the  
495 bacteria in ancient amber would have been modulated both by spores, but also by bacterial  
496 communications, notably via *luxS*. Indeed, the discovery of the *LuxS* gene, a promoter of  
497 quorum sensing detection in ancient bacteria isolated from amber stored between 25 and 40

498 million years ago, suggests that there would be horizontal transfers over millions of years  
499 between these ancient and contemporaries bacteria [152]. Some authors doubt these studies  
500 considering that DNA could not survive more than 1.5 million years in an environment [156],  
501 while these studies exclude any possibility of contamination given the precautions taken  
502 during sampling and laboratory manipulations. However, although some of these studies on  
503 amber have been the subject of much criticism [156,157], there is no tangible scientific  
504 evidence to assert or not the actual survival of these microorganisms during all these millions  
505 of years in amber.

506

## 507 **THE ANTIQUITY OF ANTIBIOTIC RESISTANCE**

508 Studies on ancient samples have shown that these samples represent an important source of  
509 discovery of the antiquity of antibiotic resistance. The most relevant samples are permafrost  
510 samples thousands and millions of years old, wherein many resistance genes have been  
511 reported. The first discovery of multiresistant strain in permafrost sediments dates back to  
512 2008 in a study conducted by Mindlin et al. revealing bacterial strains resistant to  
513 aminoglycosides, chloramphenicol, tetracycline and harboring strA-strB genes encoding  
514 aminoglycoside phosphotransferases and aadA genes encoding aminoglycoside  
515 adenylyltransferases [158]. However, other studies have confirmed that some bacteria isolated  
516 from permafrost were resistant to the majority of classes of antibiotics produced naturally in  
517 microorganisms, but also modern synthetic antibiotics such as amikacin [159]. Their genomic  
518 analyzes confirm the presence of genes (most of which are localized on plasmids), encoding  
519 resistance to streptomycin, tetracycline [160,161], Beta-lactam, Fosfomycin, Fusidic acid,  
520 MLS, Macrolide, Phenicol [162], bacitracin [163] and to heavy metals, such as mercury,  
521 cobalt, zinc, cadmium, copper, chromium and arsenic compounds [161].

522 These antibiotic resistance genes found in permafrost are sometimes followed by mobile  
523 genetic elements, such as integrons, plasmids and insertion sequences [163], the majority of  
524 which are already known, but some may also be found that have never been identified in  
525 studies of contemporary microorganisms. This is the case of a new insertion sequence named  
526 "ISPPy1" discovered in *Psychrobacter psychrophilus*, a bacterium isolated from permafrost  
527 aged 35,000 years [160]. In addition, a variety of resistance genes coding for  $\beta$ -lactam,  
528 tetracycline and glycopeptide has also been reported in metagenome data of 30,000 year old  
529 permafrost sediment [164].

530 Comparative studies of resistance profiles confirm that these resistance genes are similar to  
531 their modern counterparts and variants [160–162,164], thus stipulating a reduced evolution of  
532 these resistance genes over several thousands of years.

533

## 534 **CONCLUSIONS**

535 A wide array of methods **is** currently available to decipher microbial diversity in ancient  
536 samples (Table 3). Currently, next generation sequencing (NGS) methods provide exciting  
537 perspectives enabling both compositional and functional analyses. For a more comprehensive  
538 overview of the microbial community of the ancient samples, association of these methods  
539 could be required as a polyphasic approach. Studies on ancient microorganisms have revealed  
540 that certain metabolic pathways **have emerged** or increased over the years as **witnesses** of the  
541 evolution or adaptation to changing environments and that many **antibiotic resistance genes**  
542 have niches in ancient environments. The fact that many of the ancient bacterial genera are  
543 reported at least once in humans (Table 2) [130] underlines the importance of such studies.

544

## 545 **PERSPECTIVES ON MICROBIAL EVOLUTION**



546           Studies **on** microorganisms from ancient environments are crucial as they are currently  
547 the single ones **that can confirm or disprove the various theories of evolution**, as  
548 microorganisms in these environments can survive for millions of years, whereas such studies  
549 on human beings could not exist. In this sense, the survival of microorganisms in ancient  
550 samples can perpetuate for a considerable time, as bacterial strains that can survive in  
551 permafrost up to 3.5 million years [32,128,165], in amber dated about 120 million **years** old,  
552 [34,35] or in halite dated 250 million **years** old [146].

553           Thus, even **when** trapped by cold and salinity, microorganisms can be preserved in  
554 ancient environments **under** conditions that are unfavorable to their survival. Moreover, the  
555 melting of permafrost due to global warming is causing the reappearance of these long-  
556 trapped and preserved bacterial populations [166].The first conclusions that can be drawn  
557 from the comparison **made** between ancient and contemporary strains suggest that Darwinian  
558 Theory regarding the evolution is not relevant when applied to bacterial populations.

559           Our anthropocentric vision suggests that the human being evolves rapidly [167], but  
560 this evolutionary speed seems to be rather applicable to vertebrates than to bacteria. This is  
561 therefore a proof of the human vanity to define and delineate bacterial species which, in our  
562 view, belong to another side world. The variety of species in the visible and the invisible have  
563 in fact few in common.

564           Considering the significant number of millions of years under different conditions in  
565 which these ancient bacterial environmental species can survive, we believe that several  
566 parameters (mastered or not) are potentially **relevant** to understand what **has** kept them all  
567 these years and how they **have** evolved. It is not possible to define their molecular evolution  
568 relying on a substitution rate that would be supposed to be constant over the years [168]. In  
569 conclusion, studies on ancient microorganisms make it possible to build a real molecular

570 clock and gene network, which is neither speculative nor universal since it has long been  
571 considered a research tool [169].

572 **FUNDING**

573 This study was supported by the “Méditerranée-Infection” Foundation; program  
574 “Investissements d’Avenir”, managed by the Agence Nationale de la Recherche (reference  
575 Mediterranee Infection ANR-10-IAHU-03); Région Provence-Alpes-Côte d’Azur and  
576 European funding FEDER PRIMI.

577

578 **CONFLICT OF INTEREST**

579 The authors declare no conflict of interest

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Queries	Pubmed database	Google scholar database	Web of Science database
Ancient DNA/ Ancient microorganisms	("ancient DNA "[Tiab] AND ("culture "[Tiab] OR "metagenomics "[Tiab] OR " microscopy "[Tiab] OR " PCR "[Tiab] OR " Genomics"[Tiab])  ("ancient microorganisms "[Tiab] AND ("culture "[Tiab] OR " metagenomics "[Tiab] OR " microscopy "[Tiab] OR " PCR "[Tiab] OR " Genomics"[Tiab])	"ancient microorganisms " AND ("culture " OR " metagenomics "OR " microscopy " OR " PCR " OR " Genomics"	Ancient microorganisms
Culture Bacteria Permafrost	("bacteria"[Tiab] AND ("permafrost "[Tiab] AND ("isolation "[Tiab] OR "Culture Media"[Tiab] AND "Culture Techniques"[Tiab] OR media[Tiab] OR medium[Tiab] OR cultiv*[Tiab] OR cultur*[Tiab])	"bacteria" AND "permafrost " AND isolation OR Culture Media AND "Culture Techniques" OR media OR medium OR cultiv* OR cultur*	"Permafrost" AND "cult*"AND "bacter*"AND "microorganism*"
Molecular techniques Permafrost	("bacteria"[Tiab] AND ("permafrost "[Tiab] AND (" Metagenomics "[Tiab] OR " Molecular Diagnostic Technique"[Tiab] OR "NGS"[Tiab] OR "PCR"[Tiab] OR " Genomics"[Tiab]) OR " RNA Sequence Analysis"[Tiab])	"bacteria" AND "permafrost" AND " Metagenomics " OR " Molecular Diagnostic Technique" OR "NGS" OR "PCR" OR " Genomics" OR " RNA Sequence Analysis"	"Permafrost" AND "metagenomic*"AND "bacter*"AND "microorganism*" ; "Permafrost" AND "PCR*"AND "bacter*"AND "microorganism*"; "Permafrost" AND "genomic*"AND "bacter*"AND "microorganism*"; "Permafrost" AND "molecular*"AND "bacter*"AND "microorganism*"; "Permafrost" AND "RNA"AND "bacter*"AND "microorganism*"
Microscopy Permafrost	("permafrost "[Tiab] AND (" Microscopy "[Tiab] OR " Electron, Microscopy "[Tiab] OR " Scanning Probe "[Tiab] OR " Fluorescence, Microscopy "[Tiab])	"permafrost " AND " Microscopy " OR " Electron, Microscopy " OR " Scanning Probe " OR " Fluorescence, Microscopy "	"Permafrost" AND "microscopy" AND "bacter*"AND "microorganism*"
Mass Spectrometry Permafrost	("permafrost "[Tiab] AND (" Mass Spectrometry "[Tiab] OR " Spectrometry, Mass, Matrix-Assisted Laser Desorption-Ionization "[Tiab] OR " Spectrum Analysis mass "[Tiab] OR " Mass Spectroscopy "[Tiab])	"permafrost " AND " Mass Spectrometry " OR " Spectrometry, Mass, Matrix-Assisted Laser Desorption-Ionization " OR " Spectrum Analysis mass " OR " Mass Spectroscopy "	"Permafrost" AND " Mass Spectrometry" AND "bacter*"AND "microorganism*"
Fluorescent in situ hybridization /Permafrost	("permafrost "[Tiab] AND (" fluorescent in situ hybridization "[Tiab] OR " Staining and Labeling"[Tiab] OR " FISH "[Tiab] OR " In Situ Hybridization, Fluorescence "[Tiab])	"permafrost " AND " fluorescent in situ hybridization " OR " Staining and Labeling" OR " FISH " OR " In Situ Hybridization, Fluorescence	"Permafrost" AND " FISH" AND "bacter*"AND "microorganism*"
Multidrug Resistance Gene /Permafrost	("permafrost "[Tiab] AND ("Antibiotic resistance "[Tiab] OR " Drug Resistance "[Tiab] OR " Drug Resistance, Microbial "[Tiab] OR " Drug Resistance, Microbial "[Tiab] OR " Drug Resistance, Bacterial"[Tiab] OR " Resistance genes "[Tiab] OR " Genes, MDR "[Tiab])	"permafrost " AND "Antibiotic resistance " OR " Drug Resistance " OR " Drug Resistance, Microbial " OR " Drug Resistance, Microbial " OR " Drug Resistance, Bacterial" OR " Resistance genes" OR " Genes, MDR "	"permafrost " AND "Antibiotic resistance "; "permafrost " AND "resistance genes";



**Table 2. The most common bacterial isolates found in different samples of permafrost soils**

<b>Genera of bacterial isolates</b>	<b>Site</b>	<b>References</b>
<i>Arthrobacter</i> <i>Planococcus</i> <i>Planomicrobium</i> *	Siberia Canada China	[116,119,124,170]
<i>Arthrobacter</i> <i>Bacillus</i> <i>Paenibacillus</i> <i>Pseudomonas</i> <i>Sphingomonas</i>	Northeast Greenland Siberia Canada	[26,36,99,116,117,119,121,128]
<i>Flavobacterium</i>	Northeast Greenland Siberia	[116,117]
<i>Rhodococcus</i> <i>Streptomyces</i> <i>Cryobacterium</i> *	Northeast Greenland Canada high Arctic	[26,117,121]
<i>Sporosarcina</i>	Siberia Canada high Arctic	[26,116,121]
<i>Subtercola</i> *	Northeast Greenland Siberia	[117,128]
<i>Psychrobacter</i>	Siberia Svalbard, Arctic	[116,119,171]
<i>Aurantimonas</i> * <i>Blastococcus</i> <i>Bradyrhizobium</i> <i>Brevundimonas</i> <i>Cellulomonas</i> <i>Devosia</i> <i>Dyadobacter</i> * <i>Mesorhizobium</i> <i>Methylobacterium</i> <i>Mucilaginibacter</i> * <i>Mycobacterium</i> <i>Nocardioides</i> <i>Paenisporosarcina</i> * <i>Pedobacter</i> * <i>Rhizobium</i> <i>Sanguibacter</i> <i>Sodalis</i> * <i>Variovorax</i>	Northeast Greenland	[117]
<i>Exiguobacterium</i> <i>Psychrobacter</i> <i>Pseudomonas</i> <i>Sphingomonas</i> <i>Phenanthrenivorans</i> <i>Glaciimonas</i> * <i>Polaromona</i> *	Siberia	[27,116,119,128]
<i>Micrococcus</i> <i>Kocuria</i> <i>Marinobacter</i> * <i>Halomonas</i> <i>Sediminibacillus</i> <i>Paraliobacillus</i>	Canada	[26,36]
<i>Acinetobacter</i> <i>Enterobacter</i> <i>Nesterenkonia</i> <i>Sphingobacterium</i> <i>Sphingopyxis</i> * <i>Stenotrophomonas</i> <i>Virgibacillus</i>	Svalbard, Arctic	[171]
<i>Microbispora</i> * <i>Corynebacterium</i> <i>Streptacidiphilus</i> * <i>Frigoribacterium</i> * <i>Burkholderia</i>	Canada high Arctic	[121]

\* Bacterial genera not found in humans. All others are reported at least once in human being [130]

Table 3. Different methods of studying microorganisms in ancient samples

Methods	Principles	Advantages	Drawbacks	Applications
<b>Microscopy</b> -Transmission electron microscopy -Scanning electron microscope -Light microscopy -Epifluorescence microscopy	Rapid screening of microorganisms contained in ancient samples	It offers a quick first preview of the microbial community	It does not always determine the entire microbial community in an ancient sample	-Determination of bacilli and cocci in ancient halite samples [20,21] -Determination of bacteria in ancient teeth samples [172] -Determination of prokaryotic associated to permafrost samples [24] - Determination of the microbial morphology and biofilm formation in permafrost samples [17].
<b>Cultures techniques</b>	-Implies that microorganisms are alive in the ancient sample - Bacterial strain isolation requires appropriate culture media (enriched medium, oriented medium ...)	- It allows to have isolates of microorganisms contained in ancient samples - Contributes to understanding the biodiversity of bacterial in ancient samples	Microorganisms whose DNAs are destroyed in the sample are not detectable by culture	<i>For microorganisms culture in Siberian and Canadian permafrost:</i> -Nutrient-poor media (R2A; PYGV) [26,32] -Nutrient nutrients media (LB , TSA) enriched or not with vitamin/antibiotics [26,32] - Halophilic media [36]  <i>For microorganisms culture in Alaskan permafrost [27]</i> - Solid media Frozen  <i>For microorganisms culture in amber samples [33–35].</i> -Nutrient nutrients media (LB, TSA) enriched or not with vitamin/antibiotics  <i>For microorganisms culture in halite samples [21]</i> - Halophilic media
<b>PCR and its variants</b>	-Based on the amplification of ancient DNA molecules and depending on the case, it targets: <ul style="list-style-type: none"> <li>✓ 16S RNA generegion</li> <li>✓ ITS region</li> <li>✓ 18S rDNA</li> </ul>	- Quick - Facilitates the characterization of ancient DNAs fragments	Can amplify contaminating DNAs fragments, causing several false positives	-Study of haloarchaeal diversity [65,66] -Study of bacterial diversity [54] -Study of fungal community [66,134]
<b>Molecular cloning</b>	Amplification of the DNA fragment of interest	Minimizes the determination of	To do before DNA sequencing: prolongs	All types of ancient DNA subject to contamination by modern DNAs [73,74]

		incorrect sequences of ancient DNAs	the time and cost of ancient DNAs studies	
<b>NGS techniques</b>	Based on the entire extraction of 16S RNA genecontent from the ancient sample	-Better coverage of the microbial content range of ancient samples. -Facilitates microbial genomic studies	These techniques do not produce microbial isolates	All types of ancient DNAs [73,74]
<b>FISH</b>	Uses labeled fluorescent probes to detect nucleic acid sequences contained within the microbial cell itself at its chromosomal phases	High efficiency in the detection and quantification of microorganisms community	FISH results are influenced by the metabolic activities of the microbial cell	-Determination of bacterial community of Siberian tundra soil [50] -Quantification of in situ microbial populations in complex environmental samples [86]
<b>Microbial Detection Array</b>	Based on the use of long oligonucleotide probes	Fast and less expensive compared to sequencing techniques	Few ancient microorganisms have been reported using this technique	Detection of <i>Vibrio cholerae</i> and <i>Yersinia pestis</i> in ancient samples [43]
<b>Immunoassay techniques</b>	Monoclonal antibody assay kit	-Effective for ancient intestinal parasites study -Supplements the microscopic analysis	Few ancient microorganisms have been reported using this technique	Detection of <i>Giardia duodenalis</i> coproantigen [52] and <i>Entamoeba histolytica</i> coproantigen [53] in several coprolites
<b>MALDI-TOF MS</b>	Based on the analysis of protein profiles	Fast and effective	-All protein profiles of microorganisms are not detected -Requires a regular update of the database	Determination of a unique protein profile of a new bacterial species of the genus <i>Bacillus</i> isolated in an ancient Siberian permafrost [98,99]
<b>Metaproteomic</b>	A method of analysis of non-nucleoside biomolecules	It provides all the proteomic information contained in a complex sample	/	Determination of protein of <i>Tannerella forsythia</i> , <i>Porphyromonas gingivalis</i> and <i>Treponema denticola</i> in medieval dental calculus [101]
<b>Raman spectroscopy</b>	A non-destructive method of searching for microbial biomarker	Fast	Its use was reported only in studies on ancient halite	Identification of biomolecules of microorganisms included in the fluid inclusions of halite [102–104]

1179

**Figure List**

1180

1181 **Figure 1. Bibliographic research methodology**

Databases



Total scientific articles

166

50784

562

Selected scientific articles

58

158

87

218 scientific articles selected

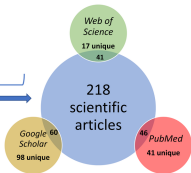
+

125 scientific articles

=

343 articles

Manual searches in web of science, google, google scholar, Pubmed:



172 relevant articles retained for the review