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ABSTRACT

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

INTRODUCTION

While the recent dramatic evolution of methods commonly used in the field of microbiology is mainly used to analyze contemporary specimen, studies on ancient samples have increased considerably in recent years. Notably, these studies clarify our understanding on the changes of microbial community at different periods as well as the associated phenomena [1,2], including climatic conditions [3,4], infectious diseases or outbreaks [5,6]. These studies are useful for understanding the history of diseases associated with significant human mortality in the past [7] or discovering the antiquity of certain diseases, long considered to be associated with modern humans [8]. In addition, studies on ancient samples provide an understanding of how individuals in microbial communities in an entire population or in an ancient environment have evolved [9–11]. Among the factors facilitating these studies, the reduction in the cost of high throughput sequencing techniques, as well as the advanced and advent of other OMICS strategies have contributed widely to the field [2,5,12].

In this review, we propose first to detail the methods used to recover ancient microorganisms through several examples and then to highlight the conclusions of environmental studies in terms of ancient microbes' diversity, through examples such as permafrost, ancient halite, amber and ancient rocks that have prompted several investigations.

BIBLIOGRAPHICAL METHODS

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

permafrost sample", "contaminant in amber sample", "contaminant in ancient rocks sample", "contaminant in ancient halite sample".

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

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

TECHNIQUES USED FOR THE DETECTION OF ANCIENT MICROORGANISMS

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

Microscopy

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

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

Culture techniques

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

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

Molecular diagnostic techniques

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

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

DNA extraction

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

Polymerase chain reaction (PCR) and its variants

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

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

✓ *Standard PCR*

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

✓ *Multiplex PCR and Quantitative PCR (qPCR)*

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

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

✓ *Nested PCR*

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

✓ *Suicide PCR*

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

✓ *Immuno-PCR*

Immuno-PCR is a technique that has been developed in 1992, which combines the advantages of immunodetection and the strong amplification and specificity of PCR, thus forming a very strong antibody-DNA complex capable of detecting very low amount of microbial DNAs [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

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

Mass spectrometry

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

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

Metaproteomic

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

Raman spectroscopy

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

Contaminants

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

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

Contaminants management

To avoid the contaminants caused by a lack of standard precautions, the authors suggested strict asepsis and antiseptic conditions consisting mainly of the use of sterile disposable equipment (wearing gloves, masks, caps, etc.), the use of a clean laboratory dedicated solely to the handling of ancient samples, systematic cleaning of all PCR equipment before handling [106]. Then, decontamination techniques on ancient samples such as permafrost , amber, ancient halite and ancient rocks samples have been proposed [61,67,110–112] to avoid any amplification of DNA from contaminants. The various decontamination protocols included: the use of ethanol and/or a Bunsen lamp; use of acid; use of bleach wash; use of combination of 6% (w/v) sodium hypochlorite saturated NaCl, NaOH, saturated NaCl, HCl, saturated Na2CO3/NaCl; use of saturated NaCl and 70% (v/v) ethanol [61,112]; use of peroxide, ethanol and heat [113,114]; use of 2% Glutaraldehyde [115].

APPLICATIONS TO DIFFERENT ENVIRONMENTAL MICROBIOMES

The environmental microbiome is filled with all types of microorganisms and certainly the oldest known microorganisms on earth [106]. Regarding environmental microbiome studies, 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,

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

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

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

diversity of permafrost (Siberian, Greenland) can be found in the active layer [131,132]. Sediments of permafrost are also often rich in methanotrophic bacteria [133], highlighting that methane can be used by bacteria living there as a source of carbon and energy for their survival in such an extreme environment. Furthermore, the microbial diversification of permafrost also widens to molds [27,120] and yeasts [27,134], whose determination is based on the analysis of internal transcribed spacer (ITS2) sequences in total DNA of sample [120], but also 18S rDNA sequences analysis [134]. The yeasts isolated in the permafrost are mainly represented by the genera *Mrakia* and *Leucosporidium* [27], known to adapt to cold environments [134–136].

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

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

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

Other environmental microbiomes

Ancient rocks

Several studies have already reported the presence of microorganisms in rocks dating from the Precambrian period (ie the period between 4.55 billion years and 540 million years). Nonetheless, very little information is available regarding the culture of microorganisms from ancient rocks. The scientist Chas. B. Lipman, first reported the presence of living bacteria living in ancient rocks, and especially in a Pre-Cambrian rock from the Algonkian in Canada [114]. In particular, rod shaped and sporulated bacteria represented the groups of bacteria 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 *Melanocyrillium*) 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

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

Amber

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

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

THE ANTIQUITY OF ANTIBIOTIC RESISTANCE

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

These antibiotic resistance genes found in permafrost are sometimes followed by mobile genetic elements, such as integrons, plasmids and insertion sequences [163], the majority of which are already known, but some may also been found that have never been identified in studies of contemporary microorganisms. This is the case of a new insertion sequence named "ISPpy1" discovered in *Psychrobacter psychrophilus*, a bacterium isolated from permafrost aged 35,000 years [160]. In addition, a variety of resistance genes coding for β-lactam, tetracycline and glycopeptide has also been reported in metagenome data of 30,000 year old permafrost sediment [164].

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

CONCLUSIONS

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

PERSPECTIVES ON MICROBIAL EVOLUTION

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

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

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

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

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577

578 **CONFLICT OF INTEREST**

579 The authors declare no conflict of interest

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1174 **Table 1. Queries used for bibliographic research**

1175

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";

1176 **Table 2. The most common bacterial isolates found in different samples of permafrost soils**

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

1177 * Bacterial genera not found in humans. All others are reported at least once in human being [130]

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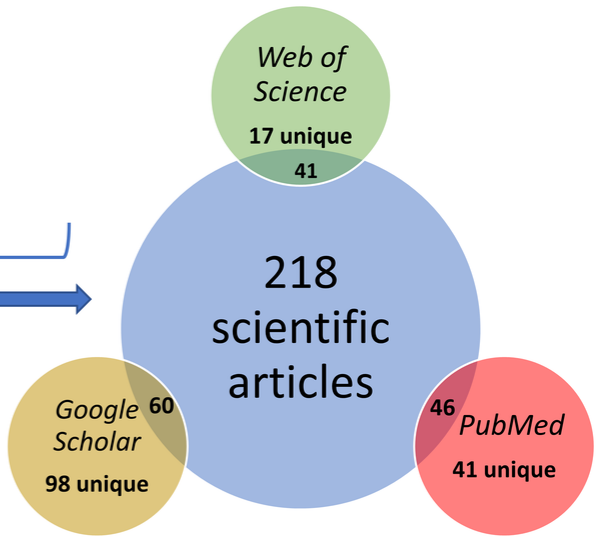
Table 3. Different methods of studying microorganisms in ancient samples

Methods	Principles	Advantages	Drawbacks	Applications
Microscopy -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].
Cultures techniques	-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	*For microorganisms culture in Siberian and Canadian permafrost*: -Nutrient-poor media (R2A; PYGV) [26,32] -Nutrient nutrients media (LB , TSA) enriched or not with vitamin/antibiotics [26,32] - Halophilic media [36] *For microorganisms culture in Alaskan permafrost* [27] - Solid media Frozen *For microorganisms culture in amber samples* [33–35]. -Nutrient nutrients media (LB, TSA) enriched or not with vitamin/antibiotics *For microorganisms culture in halite samples* [21] - Halophilic media
PCR and its variants	-Based on the amplification of ancient DNA molecules and depending on the case, it targets: ✓ 16S RNA generegion ✓ ITS region ✓ 18S rDNA	- 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]
Molecular cloning	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 ancients DNAs	the time and cost of ancient DNAs studies	
NGS techniques	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]
FISH	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]
Microbial Detection Array	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 *Vibrio cholerae* and *Yersinia pestis* in ancient samples [43]
Immunoassay techniques	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 *Giardia duodenalis* coproantigen [52] and *Entamoeba histolytica* coproantigen [53] in several coprolites
MALDI-TOF MS	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 *Bacillus* isolated in an ancient Siberian permafrost [98,99]
Metaproteomic	A method of analysis of non-nucleoside biomolecules	It provides all the proteomic information contained in a complex sample	/	Determination of protein of *Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola* in medieval dental calculus [101]
Raman spectroscopy	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]

Figure List

Databases	WEB OF SCIENCE	Google Scholar	PubMed
Total scientific articles	166	50784	562
Selected scientific articles	58	158	87



218 scientific articles selected

+

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

125 scientific articles

=

343 articles

172 relevant articles retained for the review