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BIOMOLECULAR CHARACTERIZATION OF THE ANCIENT MICROBIOME IN ARCHAEOLOGICAL SAMPLES IN LATVIA

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Abstract

The area of ancient human microbiome research has evolved rapidly over the past few decades and has become a topic of great scientific interest. Modern sequencing technologies enable us to access the valuable information about historic microbial communities, providing us important insights into anthropological questions of human history and evolution, such as our ancestral lifestyle, health and diseases. Until now there have been no studies on historic microbiome in archaeological samples from Latvia. Within my work, I have investigated ancient DNA (aDNA) datasets and microbiome composition of human postmedieval archaeological bone, tooth and dental calculus samples in Latvia, Northern Europe, dated 15th–17th century AD.

Significant infiltration and contamination of archaeological bone and tooth samples with environmental microbial species was observed, while the majority of microbial DNA in historic dental calculus originated from the oral microbiome with little impact of the burial environment. Possible microbial traces of soft tissue decomposition profiles within human bones were detected, which makes archaeological human bones a potentially useful material for historic necrobiome research. Microbial data obtained from historic dental calculus samples provided a reliable snapshot of oral bacterial communities from past individuals. Historic Latvian dental calculus specimen data also proved the existing hypothesis stating that bacterial communities of different oral cavity's formations carry significant differences. Several potentially pathogenic bacterial species were identified within the samples of historic dental calculus. In addition, important methodological question of aDNA authentication was studied along with the factor of environmental bacterial influence on the specimens. Apart from microbial DNA, a relatively good yield of endogenous human aDNA was discovered in human postmedieval archaeological samples, which enables future studies on early-modern historic Latvian populations.

Overall, this work explored the historic human-related microbiota, preservation of ancient biomolecules in different postmedival archaeological samples, and a possible impact of the burial environment and environmental DNA on ancient microbiome reconstruction. Accumulating ancient microbiome-related data contribute to the progress of this research area.

Abstrakts

Senā cilvēka mikrobioma izpētes joma pēdējo gadu laikā ir strauji attīstījusies un kļuvusi par tematu, kas rada lielu zinātnisku interesi. Mūsdienu sekvencēšanas tehnoloģijas ļauj mums piekļūt vērtīgai informācijai par vēsturiskām mikroorganismu kopām, sniedzot svarīgu ieskatu cilvēces vēstures un evolūcijas jautājumos, piemēram, par mūsu senču dzīvesveidu, veselību un slimībām. Līdz šim nav veikti pētījumi par vēsturisko mikrobiomu Latvijas arheoloģiskajos paraugos. Savā darbā esmu pētījusi senās DNS (aDNS) datu kopas un mikrobioma sastāvu arheoloģiskos pēcviduslaiku cilvēka kaulu, zobu un zobakmens paraugos Latvijā, Ziemeļeiropā, kas ir datēti ar mūsu ēras 15.–17.gadsimtu.

Arheoloģisko kaulu un zobu paraugiem tika konstatēta ievērojama infiltrācija un piesārņojums ar vides mikrobu sugām, savukārt lielākā daļa mikrobu DNS vēsturiskajos zobakmeņos radās no mutes mikrobioma ar nelielu apbedījuma vides ietekmi. Tika atklātas iespējamās mīksto audu sadalīšanās procesos iesaistītu mikrobu pēdas cilvēka arheoloģiskos kaulu materiālos, kas padara šos paraugus par potenciāli noderīgu materiālu vēsturiska nekrobioma pētījumiem.

Mikroorganismu DNS dati, kas iegūti no vēsturiskiem zobakmeņu paraugiem, sniedza ticamu informāciju par mutes dobuma baktēriju kopām no pagātnes indivīdiem. Vēsturiskie Latvijas zobakmens paraugu dati arī pierādīja pastāvošo hipotēzi, ka dažādās mutes dobuma daļās esošās baktēriju kopas ir būtiski atšķirīgas. Vēsturiskos zobakmens paraugos tika identificētas vairākas potenciāli patogēnas baktēriju sugas. Tika pētīts arī svarīgs metodoloģiskais jautājums par aDNS autentifikāciju, kā arī par iespējamo vides baktēriju ietekmi. Pētamajos cilvēka pēcviduslaiku arheoloģiskajos paraugos papildus mikroorganismu DNS tika atklāts arī salīdzinoši labs endogēnās cilvēka aDNS daudzums, kas ļaus turpmākos pētījumos raksturot vēsturiskās Latvijas populācijas.

Kopumā šajā darbā tika pētīts vēsturiskais cilvēka mikrobioms, novērtēta seno biomolekulu saglabāšanas pakāpe dažādos pēcviduslaiku arheoloģiskajos paraugos un raksturota iespējamā apbedījumu vides mikroorganismu DNS ietekme uz seno mikrobiomu rekonstrukciju. Ar seno mikrobiomu saistīto datu uzkrāšana veicina šīs pētniecības jomas progresu.

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Abbreviations

16S – mitochondrial ribosomal RNA

aDNA – ancient DNA

DNA – deoxyribonucleic acid

eDNA – environmental DNA

HMP – Human Microbiome Project

LCA – lowest common ancestor

NGS – Next Generation Sequencing

OTUs – operational taxonomic units

PCA – principle component analysis

PCR – polymerase chain reaction

RNA – ribonucleic acid

rRNA – ribosomal RNA

UV – ultra violet

Introduction

Microbial cells in human body outrange the number of native human cells by 1.3x (Gilbert et al., 2018). Furthermore, there are 100 times more bacterial genes in our body compared with our own genes (Yang et al., 2009). The study of human microbiome – ecological community of all microorganisms from a specific site or area of human body – underwent accelerated growth worldwide during the last few decades (Lederberg and Mccray, 2001). Advances in genomics, such as Next Generation Sequencing (NGS) technology, contributed to this growing interest, and the Human Microbiome Project (HMP) was launched in 2007 with the main focus on the identifying and characterizing human microbial flora (https://hmpdacc.org/). HMP and other recent studies have generated new knowledge to begin to identify properties and functions of the host microbiome. It is widely recognized that the microbiome plays a pivotal role in human biology, including the production of important resources, bioconversion of nutrients, and protection against pathogenic microbes (Young, 2017).

It has also become increasingly clear that the study of human evolution is not complete without understanding of microbiome evolution and changing ecology through time, and advances in NGS allowed us to access microbial information from historic human remains specimens, opening up the field of ancient human microbiome research (Orlando et al., 2021). Discovering our past microbial self has contributed to answering many important modern-day health related questions, as well as anthropological questions of human history and evolution. For example, ancient gut microbiome studies provided us a notion of previously unknown ancestral gut microbial diversity which is appears to be lost nowadays together with some key microbial symbionts (Tett et al., 2019; Tito et al., 2008). These and similar ancestral gut microbiome studies have contributed to the formation of a widespread hypothesis, linking modern western chronic diseases to gut microbial diversity loss (Wibowo et al., 2021). Given that diet is a shaping factor of oral microbiome composition, ancient oral microbial studies have provided us direct evidence of some major ancestral dietary shifts like Neolithic and Industrial revolution, introducing carbohydrate-rich foods (Warinner et al., 2015b). Furthermore, historic human-related microbial studies allowed scientists to access the genetic information of ancient microbial pathogens including those that may leave characteristic lesions on host bones, and those that leave no visible evidence. Such findings contribute to pathogen evolution studies and also could solve historic mysteries around the actual causes of ancient epidemics (Orlando et al., 2021). Studies of historic human microbiomes and historic environmental microbiomes have shed light on important modern question of microbial antibiotic resistance by providing the evidence of antibiotic-resistance gene presence in ancient specimens. Based on these findings a hypothesis was formed

stating that many modern antibiotic-resistance genes are most likely of the environmental origin (Perry et al., 2016). Also, studies of microbial colonization of bones during the postmortem skeletal degradation could provide important insights into possible factors related to the postmortem human DNA degradation (Emmons et al., 2020).

Taken together, ancient human microbiome research paves the way for evolutionary studies of an assemblage of human and many other species living in or around it, which, in its turn, can significantly contribute to our understanding of historic social, dietary and environmental shifts and reveal factors that are influencing individual and population health today.

Importance of this work

This work is highly relevant in the context of archaeological biomolecule research as it provides insights into historical microbiome composition of human postmedieval archaeological bone and tooth samples in Latvia, Northern Europe, dated 15th–17th century AD. It is also relevant for historic Latvian population studies, by providing novel data on ancient oral microbiome communities. Overall, this Thesis widens our knowledge on historic human-related microbiota, as well as explore the preservation of ancient biomolecules in different postmedival archaeological samples, and highlights a possible impact of the burial environment and environmental DNA on ancient microbiome reconstruction. Accumulating ancient microbiome-related data contribute to the progress of this research area.

Aims of the study

- To explore historic microbiome communities in human postmedieval archaeological bone, tooth and dental calculus samples in Latvia, Northern Europe, dated 15th–17th century AD.
- To explore the degradation profiles of aDNA in postmedieval archeological samples in Latvia and to evaluate a possible impact of the burial environment.

Tasks to reach the aims

- Perform analysis of the microbiome profiles of human postmedival archaeological bone samples in comparison with the corresponding soil samples of the burial environment.
- Investigate the microbiome profiles of historic postmedival dental calculus samples in comparison with modern dental calculus, dental plaque samples and burial soil microbiota.
- Evaluate the preservation of human oral microbiome patterns and aDNA in historic postmedival dental calculus samples;
- Evaluate aDNA preservation and taxonomic diversity in postmedieval human tooth samples and explore the presence and diversity of environmental bacteria in aDNA datasets.

1. Literature overview

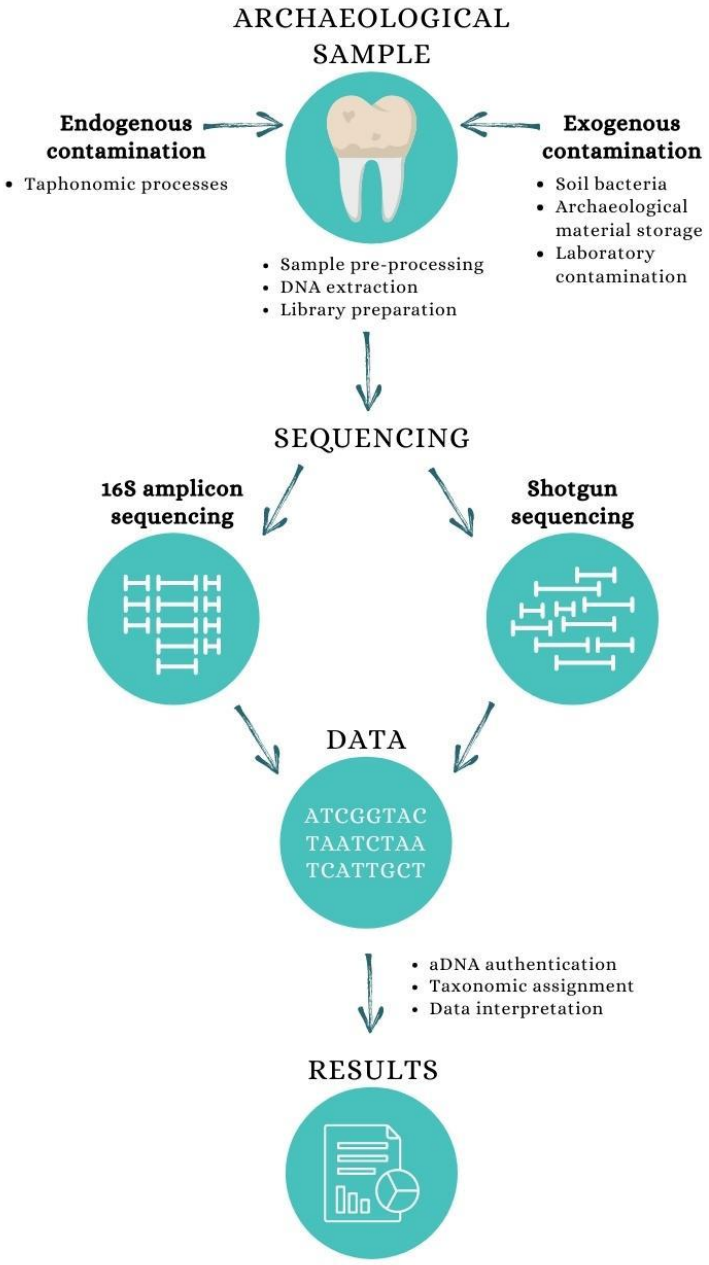
1.1. History of aDNA research and standard research workflow.

Ancient DNA research era started in 1984, when for the first time DNA fragments were succesfully extracted and sequenced from dried muscle of museum specimen - extinct zebra species (Higuchi et al., 1984). Four years later, in 1988, mitochondrial DNA fragments from 7000-year-old human brain were extracted, amplified using polymerase chain reaction (PCR) and sequenced (Pääbo et al., 1988). Soon after the PCR technique has become the method of choice when working with aDNA, and in further years numerous researches were carried out to study aDNA molecules in human archaeological samples (Hagelberg et al., 1989), the remains of extinct animals (Cooper et al., 1992; Thomas et al., 1989, 1990), and historic fossilizes plants and insects (Cano et al., 1993; Golenberg et al., 1990; Hagelberg et al., 2015). In addition to the host DNA, advances in genomics opened up a new frontier for ancient pathogen research (Salo et al., 1994). Furthermore, PCR-based methodology also allowed to explore the diversity of ribosomal RNA (rRNA) sequences from multiple environments, opening the path for metagenomics – study of a bulk microbial DNA directly from environment without the need for cultivation (Schmidt et al., 1991; Stein et al., 1996). However, first metagenomic analysis of ancient material (palaeofaeces) took place only in 2008, after the introduction of Next Generation Sequencing (NGS) techniques (Tito et al., 2008). Further decade was characterized by the exponential growth of the field of aDNA and historic microbiome research. Currently, application of NGS-based 16S rRNA profiling and shotgun sequencing are widely used to study microbiome composition in various archaeological human remains material types. Although sample-specific research design nuances may vary, procedures common for historic microbiome research usually make the basis of any related study (Diagram 1).

While novel data piled up during the beginning of ancient microbiome research era, several challenges and pitfalls in aDNA research were also identified. In particular, questions of aDNA laboratory setup, material handling, contamination control, and data analysis gained special importance (Orlando et al., 2021). Various techniques were developed to reduce sample contamination and enhance the accuracy of results (Boessenkool et al., 2017; Gamba et al., 2016; Gansauge and Meyer, 2014; Korlević et al., 2015; Orlando et al., 2021). This advance in technology along with the growing interest in aDNA studies had further shaped the research field by introducing highly specialised aDNA workstream processes needed to support the standard criteria of authenticity for validated discoveries. In general, the standard steps of historic microbiome research workflow could be summarized as following: sample pre-processing (which includes contamination control), DNA extraction, NGS library preparation, which is followed by NGS sequencing and data analysis (aDNA authentication, taxonomic assignment, data interpretation) (Diagram 1).

The literature overview sections 1.1.1. to 1.1.5. will further cover Diagram 1 in more detail.

Diagram 1. Standard workflow steps in historic microbiome research and the most important influential factors.



1.1.1. Archaeological material overview

Archaeological human remains sample types and their specific features.

Archaeological material is a fragile and precious source of historical information. Every archaeological entity bears in itself a vast amount of diverse information scattered over a wide number of topics: cultural, evolutionary, biological, sociological and many more. It is important to understand the major types of archaeological human remains material and their properties, as this information is crucial while interpreting any archaeological specimen-drown data, especially historical biomolecule data.

Three major types of biomolecules are available in archeological remains: nucleic acids, proteins and lipids (Cappellini et al., 2018). While ancient lipids and proteins are indispensable in bioarchaeological studies and, each with their specific characteristics, reveal much about ancient diet and lifestyle (Hendy et al., 2018), DNA is the most important biomolecule found in archaeological specimens, leading us to highest resolution evolutionary information and closest insights (Cappellini et al., 2018, Slatkin and Racimo, 2016). However, there are many specific nuances that must be considered to ensure correct data interpretation, especially if the subject of the study is not the host DNA but host-related microbiome. One of the parameters is DNA degradation, which is believed to have various rates depending on tissue and environmental processes around the specimen (Kistler et al., 2017). Furthermore, to capture the correct snapshot of a specific location's microbiome it is necessary to overcome two types of potential contamination. Endogenous contamination arises from same organism's different tissues and mostly is due to taphonomic processes, which dramatically transform human microbiome's ecology soon after death due to processes of soft tissue decomposition (Morris et al., 2006). Exogenous contamination, in its turn, comes from all possible exogenous sources that come in contact with the specimen of interest starting with burial soil, archaeological excavation process, archaeological sample storage, and laboratory contamination (Eisenhofer et al., 2019, Eisenhofer and Weyrich, 2018, Llamas et al., 2017). Every archaeological human remains material type represents a specific combination of the above parameters, depending on body site, anatomical features and specimen preservation properties.

Christina Warinner in her article "Ancient human microbiomes" (2014) suggested that there are five main archaeological human remain sample groups that potentially can be used for ancient microbiome reconstruction. Among them, coprolites and dental calculus are considered to be the main source of ancient human microbiome data, followed by three additional sources serving as a secondary deposit for human bacteria such as historic medical specimens, mummified human remains and human bones (Warinner et al., 2015b). Although this division is reasonable, it would also make

sense to position human teeth and tooth root archaeological material in a separate group as this material possesses some unique properties. Therefore, in this work, each sample group will be considered and explored separately.

Term “coprolites” is being used to describe desiccated and fossilized ancient feces (Reinhard and Bryant, 1992). Representing human gut microbiome, coprolites give us potential access to the most advances source of bacterial diversity, associated with living organism (Lozupone et al., 2012; Yatsunenko et al., 2012). Although coprolites are indeed one of the most biologically informative archaeological material, and evidence shows that they can survive for millions of years under favorable conditions (Dentzien-Dias et al., 2013), coprolite specimens are associated with a list of challenges and drawbacks, making them quite a complicated material to work with. First complexity factor of coprolites as a bioarchaeological material is its sensitivity to environmental conditions. Being an extremely bioactive substance, feces can preserve well and eventually turn into coprolites only in dry and cold environments (Sharma, 2005). Some studies consider them to be rare archaeological finds, while others argue that they are as abundant as other archaeological artefacts, although a person must be trained well to find them (Shillito et al., 2020). Indeed, coprolites are most often hard to identify due to their fickle appearance – they come in a variety of different shapes and textures due to very different diets and physiological and lifestyle features of humans of the past (Reinhard and Bryant, 1992). Moreover, even after the artefact has been identified as coprolite, it is still a challenge to distinguish whether it is of human origin (Reinhard and Bryant, 1992). The only undoubtedly human coprolites are the ones that are recovered from mummified human remains and burial sites (Reinhard and Bryant, 1992). Most often coprolites originate from public latrine areas (Warinner et al., 2015b) which allows to research historic human microbiome on population level but eliminates the possibility to study individual microbiome of humans of the past. One more drawback of coprolites as a bioarchaeological material for ancient microbiome studies is that they can be considered to be *open* systems, being extremely vulnerable for external contamination and therefore exhibiting screwed microbiome profile which has to be interpreted with great care (Warinner et al., 2015b).

Dental calculus is another highly informative archaeological material that is used in biomolecule research. Dental calculus is an oral plaque biofilm that underwent mineralization processes and turned into a cement-like substance in terms of both adhesive strength (Watts and Combe, 1981; White, 1997) and physical hardness (White, 1997, 1991); most importantly, it has an excellent oral microbiome preservation abilities (Adler et al., 2013; De La Fuente et al., 2013; Mann et al., 2018; Warinner et al., 2015b, 2014; Weyrich et al., 2017). It contains traces of all oral ecosystem-typical elements, which makes it a highly demanded archaeological find. In terms of

chemistry, largest proportion of dental calculus constituents are of inorganic nature – calcium and phosphorus being the leading elements, followed by magnesium, silicone, iron, fluoride and several minerals (Hayashizaki et al., 2008; Kinaston et al., 2019; Lieverse, 1999; White, 1997). Organic components constitute around 15-20%, among whose there are phytoliths, starch granules, variety of biomolecules and bacteria (Kinaston et al., 2019; Lieverse, 1999). The unique property of dental calculus is that it turns into fossil during organism's lifetime, and at the time of organism's death it is already biologically inert enough not to succumb to internal and external contamination factors such as microbiome shifts during taphonomic processes or invasion of soil bacteria. Because of these reasons, recent studies suggest that dental calculus might be the most coveted archaeological material for ancient microbiome research, allowing lowest environmental contamination and exhibiting highest aDNA yields (Mann et al., 2018; Ozga et al., 2016; Warinner et al., 2014). Dental calculus also allows differential analysis of historic oral microbiome providing individual-scale data, as well as holds information about a diverse range of oral cavity's opportunistic pathogens which might be used to trace back historic infectious disease records (Warinner et al., 2014). One significant drawback of dental calculus as a tool to study ancient human microbiome is that dental calculus gives us insights of a narrow field of oral microbial communities, slightly expanding in the directions of upper respiratory tract and upper digestive tract microbial communities, whose traces can sometimes be identified in dental calculus debris (Huynh et al., 2016; Weyrich et al., 2015). Although it is an immensely important piece of the puzzle in the concept of diverse and turbulent human microbiome, these data could be hardly extrapolate to get insights into the organism's functions and biological processes in general. Deep learning techniques are beginning to be used in the field of microbiome studies, and chances are that this rapidly emerging technology in the nearest future might expand the amount of information obtained based on microbial composition of oral cavity (Cartwright, 2021).

Historic human bone specimens is another very important type of archaeological material that is widely used due to its DNA preservation abilities. In contrast to coprolites and dental calculus, which are mostly popular in the field of ancient human microbiome studies, archaeological human bones are usually used as a source of endogenous aDNA. Nucleic and mitochondrial DNA can indeed be successfully isolated from archaeological bone material, however, again, the DNA preservation is highly dependent on various factors such as bone type and environment of decomposition (Allentoft et al., 2012; Andronowski et al., 2017; Damgaard et al., 2015; Mundorff and Davoren, 2014). One of significant environmental factors that can affect endogenous aDNA preservation in bone marrow is bacterial activity on the site (Burger et al., 1999; Elsner et al., 2015; Emmons et al., 2020). Currently, the types and distribution of bone colonizing microbes are not yet clearly understood, however, it is known that bone colonization by environmental microbes do occur (Emmons et al., 2020). On the

other hand, body decomposition is a mosaic system where both intrinsic and extrinsic bacteria are involved, thus it could be hypothesized that individual's microbes from different tissues may infiltrate bones shortly after an individual's death and remain trapped inside (Bell et al., 1996; Morris et al., 2006). Therefore, to some extent, osteological material could represent some traces of historic human microbiome. Apart from the source for historical endogenous aDNA, human-associated microbial DNA and historical and modern environmental DNA, bone material is also used in studying of blood-borne pathogens that leave diagnostic bone lesions, such as *Mycobacterium tuberculosis, Mycobacterium leprae, Yersinia pestis, Treponema pallidum* (Spyrou et al., 2019).

Considering whole teeth and tooth roots as a source for aDNA it is important to note that this material is protected from various environmental conditions far better than any other archaeological human remains material due to hard enamel and cementum layers (Adler et al., 2011; Melchior et al., 2008). Endogenous DNA levels within well-preserved historic human teeth can often be compared with those isolated from human petrous bone, which makes teeth a useful material in human aDNA studies (Hansen et al., 2017). It has also been reported that archaeological teeth samples may serve as a reservoir of human microbiota traces (Warinner et al., 2014). Ancient pathogenic bacteria have been detected in teeth throughout several studies due to the fact that teeth are directly exposed to blood-borne pathogens (Drancourt et al., 2005, 1998; Rasmussen et al., 2015; Warinner et al., 2014). Overall chances of finding an ancient pathogen in teeth sample are considered to be higher than in petrous bone sample of same individual, as was exampled by the reproducible detection of *Yersinia pestis* DNA in teeth samples of several human skeletons dated to the Bronze Age and Iron Age (Margaryan et al., 2018). Historic teeth specimens are also known to capture traces of plant and animal DNA thus providing information about our ancestral lifestyle and diet (Sawafuji et al., 2020; Warinner et al., 2014; Weyrich et al., 2017). Ancient dental pulp is also capable of capturing and storing distinct human oral microbiome taxa which makes historic teeth a potential material in ancient human microbiome studies (Margaryan et al., 2018; Rascovan et al., 2016).

Medical specimens of human tissues is yet another potential source of historic human microbiome information. Nevertheless, it is worth to consider two main limitation of this material. First, such specimens are usually limited to the past few centuries. Secondly, anatomical human material within these samples is usually preserved in formaldehyde, liquid alcohol, or stored in formalin-fixes paraffin-embedded blocks, which means that whole microbiome reconstruction scene most probably will be screwed in both quality and quantity (Gilbert et al., 2007; Warinner et al., 2014). At the same time, there is a precedent of successful historic pathogen recovery from this type of sample: causative agent of cholera, *Vibrio cholera*, has been recovered from 1849 CE alcohol-preserved medical specimen of colon in 2014 (Devault et al., 2014). This hints to the fact that historic

medical specimens can be used to trace back certain aspects of historic human microbiome and could be especially useful providing access to microbes of soft tissues, which could not be preserved (Warinner et al., 2015b).

Mummified human remains, although exhibiting apparent visual preservation of individual tissue material on a macroscopic scale, still are subjected to a full-fledged cascade of taphonomic processes, leaving molecular and bacterial scene of the tissues wildly disrupted. Although for the most body sites, it is hard to extrapolate precise scene of ancient human microbiome relying on mummified remains microbiome data in general, they still provide an outstanding opportunity to access bacterial communities left within soft tissues, which would be decomposed otherwise. For example, many studies were conducted using mummified human remains intestinal material with the intention to access historic human gut microbiome information and throughout these studies authentic gut microbial communities were identified (Rollo et al., 2007; Santiago-Rodriguez et al., 2015). Mummified human remains can also be used for historic pathogen tracking. For example, Neukamm et al. (2020) successfully reconstructed 2200-year-old *Mycobacterium leprae* genome and 2000-year-old human hepatitis B virus using bone and soft tissue of Egyptian mummified individuals (Neukamm et al., 2020).

1.1.2. Challenges in sample pre-processing and aDNA extraction

Although each archaeological material type represents unique characteristics, general handling challenges remain the same for all specimens. Throughout aDNA (and specifically: ancient microbiome) research history, many standard procedures were approved as necessary actions that should be implemented in order to minimize risks of contamination and data bias. While the endogenous sample contamination is impossible to eliminate because it took place during the process of taphonomic organic tissue degradation, there are possibilities to consider and target various exogenous contamination factor manifestations in resultant metagenomics data.

Controlling modern contamination is crucial not only because it may resemble authentic aDNA and thus lead to misinterpretation of results, but also because it may outcompete aDNA during PCR amplification reactions of NGS library preparation process and therefore critically minimize aDNA presence in theresultant data (Fulton and Shapiro, 2019). There are numerous ways how external contamination can be introduced to the sample, including soil bacteria of burial environment, human and human-associated bacteria during excavation process, environmental and cross-contamination if the samples are stored close to each other on museum shelves. Furthermore, as the samples reach laboratory, new contamination factors are here to come. DNA extraction and sequencing library

preparation stages can introduce numerous contaminants into the sample. Human DNA might be introduced by laboratory personnel, reagents and equipment might carry alien DNA fragments, even the air supply system of the building where the laboratory is located, can contribute to historic sample contamination with modern DNA, which, accordingly, will appear as a challenge factor during upcoming result analysis and interpretation. Fighting laboratory contamination is in some sense quite a quest because of the fact that contaminant DNA is intangible to the finest grade. It can be floating around in an aerosolized drop of water, which escaped the lid of opened test-tube and even 0.005 mkl of this drop can potentially contain thousands times more DNA than the historic sample of interest (Fulton and Shapiro, 2019; Leonard et al., 2007; Willerslev and Cooper, 2005).

In the recent years, numerous protocols on historic sample handling and processing were created (Cooper and Poinar, 2000; Pääbo et al., 2004; Shapiro et al., 2019; Willerslev and Cooper, 2005). In general terms, major requirements demand aDNA studies to take place in specially equipped, physically isolated facilities. Ideal circumstances would include altered air pressure in aDNA facilities: higher pressure in historic sample handling rooms and lower pressure in rooms where modern DNA handling takes pace. Historic specimen handling facilities must be routinely sterilized on a regular basis using both chemical disinfectants and UV radiation. An important requirement is to keep aDNA facilities separated from PCR-amplification facilities where millions of molecules are being created by amplification, as it is extremely difficult to avoid their spreading. Historic DNA facility's ventilation system must be separated from central laboratory's ventilation system, and aDNA personnel should contribute to the precaution practices by wearing gloves, masks and protective clothing, which ideally is stored in a separate gateway chamber on the way to aDNA facility (Grigorenko et al., 2009).

Historic sample pre-processing manipulations usually begin with removal of the surface layer with a subsequent sterilization with UV radiation (Grigorenko et al., 2009). Sodium hypochlorite is also advocated for historic sample pretreatment as a useful tool for DNA contamination removal (Korlević and Meyer, 2019). Sample powder predigestion using EDTA-based lysis buffer and Proteinase K was suggested as less aggressive sample pre-treatment step (Schroeder et al., 2019). All pre-treatment approaches are based on the idea that exogenous DNA connection to the sample's material is much weaker than connection of endogenous DNA, so it is possible to remove exogenous DNA without disrupting the authentic endogenous DNA. Usually aDNA study design involves one or several sample pretreatment steps depending on various research factors and circumstances.

Likewise, the topic of aDNA extraction has rose a range of guidelines and protocols. Although the basis of aDNA extraction mostly remains to be phenol-chloroform method, nuances depend on

many factors like sample material, age and environment of preservation (Dabney and Meyer, 2019; Hagan et al., 2020; Matsvay et al., 2019). Apart from fragile DNA molecules, another risk factor to be overcome is presence of PCR inhibitors which are numerous and usually extracted together with DNA. Humic, fulvic acids, tannins and phenolic compounds – examples of PCR inhibitors in aDNA studies – in last decade are mostly being replaced by the use of silica-based column extraction protocols (Dabney and Meyer, 2019; Matsvay et al., 2019; Rohland and Hofreiter, 2007a, 2007b).

Harvested microbiome data, by a large degree, is influenced by sample's extracted DNA quality, which, in its tern, is correlated with DNA damage scale. One of most well-known aDNA characteristics is its size – ancient DNA usually represents short, degraded fragments, generally between 40 – 300 bp (Kircher, 2012). Short aDNA fragments are further made even more challenging by accumulated damage patterns. DNA is exposed to various damage patters during its lifespan. Most common types are DNA base deamination, depurination, oxidation and methylation followed by two crosslink types: interstrand crosslink and DNA-protein crosslink (Swift and Golsteyn, 2014). Within a living organism under normal circumstances, DNA damages are supposed to be repaired via numerous intracellular mechanisms. Upon organism's death, these mechanisms stop functioning. DNA damage process, however, persists and even accelerates, being supported by various environmental catalysators. Many damage patterns that accumulate within DNA strands during its contact with various environments act as PCR inhibiting factors (Pääbo et al., 1989). Hydrolytic activity upon DNA results in three damage types: hydrolysis of phosphodiester backbone (rarely), hydrolytic deamination (most commonly: cytosine deamination, causing C to T transition) and hydrolysis of glycosidic bonds (purine bases are most exposed to this reaction; subsequently, is it called depurination and purine base is lost as a result). Single-strand breaks are known to be the result of hydrolytic DNA damage (Fulton and Shapiro, 2019; Lindahl, 1993). Oxidation results in damage patterns that most commonly block polymerases. This leads to either amplification stop or chimeric sequence production via "jumping PCR" (Fulton and Shapiro, 2019; Lindahl, 1993; Paabo, 1989; Pääbo et al., 1989). Cross-link DNA damage lesions also block polymerase chain reaction (Paabo, 1989).

1.1.3. Library preparation and sequencing

Microbiome of any biotope combines members of various domains: prokaryotes (bacteria, archaea), eukaryotes (fungi, protists), viruses (Lederberg and Mccray, 2001). At the same time, primary focus of microbiome studies in almost all fields is inevitably bacterial (Hooks and O'Malley, 2020). There are various reasons for that: bacteria is metodologically more approachable, bacterial

genes outnumber any other domain member genes (Qin et al., 2010), and bacteria are responsible for the greatest part of active metabolites that influence ecology of any community as well as dictate relationships with the host (Postler and Ghosh, 2017). Although number of eucaryotic microbiome studies began to rise in recent years, bacterial research remains in the focus of any microbiome studies to this day, including ancient microbiome research (Hooks and O'Malley, 2020). At the moment the field of microbiome research (both modern and ancient) is dominated by two main sequencing approaches: 16S ribosomal RNA (rRNA) gene amplicon sequencing and shotgun metagenomics sequencing (Jovel et al., 2016; Mas-Lloret et al., 2020; Ranjan et al., 2016; Warinner et al., 2017; Ziesemer et al., 2015). Both approaches represent the field of metagenomics – a relatively young method that, with the help of NGS, has revolutionized the study of microbial communities allowing to reach their genetic data directly from their natural environment, without the need of culturing (Shah et al., 2010; Wooley and Ye, 2010).

16S rRNA gene sequencing approach appeared earlier then shotgun sequencing approach – it has been used historically since the middle 1990s (Muyzer et al., 1993; Shah et al., 2010). The method is based on the fact that 16S rRNA encoding gene is a highly conserved gene for bacteria and archaea, allowing to access microbial phylogenesis without having to deal with non-microbial DNA admixture. The gene is built of nine hypervariable species-specific regions (V1 – V9) with highly conserved regions located between them. Usually 16S rRNA hypervariable region amplification allows to differentiate microbial communities down to genus level, however in case if the whole 16S sequence is present the differentiation is possible up to the level of species (Mas-Lloret et al., 2020). 16S sequencing is a relatively cheap and accessible method that enables simultaneous sequencing of thousands of individual 16S rRNA genes – these factors had a huge influence making Human Microbiome Project succeed (NIH HMP Working Group et al., 2009; Warinner et al., 2017). Speaking in the context of historic DNA research, there were numerous studies detecting 16S rRNA gene presence in historic samples of different age raging up to 5300-year-old ones, proving that these genes can indeed be preserved in archaeological samples and successfully retrieved from them (Arning and Wilson, 2020; Cano et al., n.d.; Ubaldi et al., 1998).

On the other hand, 16S rRNA sequencing is also associated with numerous limitations. For example, although there are nine hypervariable regions, they amplify differently for different bacteria, not a single one of them is capable to distinguish among all bacteria and conserved regions are also not entirely identical for various bacteria and archaea (Chakravorty et al., 2007; Mas-Lloret et al., 2020; Shah et al., 2010). Another notable factor is that PCR amplification of 16S rRNA gene often produces artifacts like chimeric sequences, which, according to Ashelford K. E. et al. study, are found to be present widely over 16S rRNA public repositories (Ashelford et al., 2005). These sequencing

errors may further negatively influence accuracy characterizing sample's microbial diversity (Quince et al., 2009; Shah et al., 2010). Furthermore, to deeply analyze a sample's microbial community using 16S rRNA method we would require long well-preserved 16S fragments to be present in the sample. As we already discussed earlier, aDNA usually lacks such a degree of preservation, which subsequently lowers the accuracy of 16S rRNA sequencing. Also, 16S rRNA approach can be considered highly sensitive to background contamination as to successfully target amplification, historic samples usually require a large number of PCR cycles (Warinner et al., 2017).

While 16S sequencing relies on specific 16S rRNA gene primers to capture exclusively bacteria and archaea, shotgun sequencing approach (aka whole-metagenome shotgun analysis) uses universal primers with the goal to sequence all the existing DNA molecules trapped in a sample of interest (Janda and Abbott, 2007; Jovel et al., 2016). Shotgun sequencing method has gained a wide popularity over the past decade due to its unambiguous advantages. One of the major advantages of shotgun sequencing is believed to be the fact of more accurate taxa definition at the species level (Ranjan et al., 2016). Further advantages include the possibility to dive even deeper into taxonomic representation and in some cases be able to detect specific strain of a particular species as well as the possibility to assemble metagenomes de-novo, possibility to sequence a genome in high-coverage and to perform functional characterization of retrieved metagenomic material (Mas-Lloret et al., 2020; Ranjan et al., 2016). The last shotgun sequencing specification is of a special importance in the field of ancient microbiome research as sometimes community structure and its functional potential might store much more valuable information than a list of microorganisms itself (Warinner et al., 2017). However, using shotgun sequencing for microbiome research has its drawbacks as well. Due to microbiome community complexity, shotgun metagenomics data requires most profound analysis tools and approaches in order to be correctly interpreted (Warinner et al., 2015a). The reason for this is that shotgun metagenomics datasets typically represent low sequencing coverage. As a result, microbiome reconstruction is usually limited to highly abundant taxa, leaving low-coverage taxa underrepresented (Kuczynski et al., 2012; Scholz et al., 2012).

Overall, both 16S rRNA and shotgun sequencing methods have their unique advantages and drawbacks, and careful study design must be implemented in every particular situation.

1.1.4. aDNA authentication challenges

As soon as samples of interest are sequenced (their DNA data is translated into digital data), the next step of ancient microbiome research journey begins. After initial quality control processes it is crucial to examine outcome data on the subject of aDNA authenticity.

Firstly, mandatory extraction and PCR controls with no DNA (negative controls) should be processed alongside with the samples in order to clear resultant data from laboratory and cross-contamination. There are several *in silico* approaches to clear sample data of contamination using negative controls, one of them refers to R package decontam – statistical package, evaluating each microorganism in terms of probable contamination (Davis et al., 2018). After processing negative controls, aDNA *in silico* authentication may begin.

As was already mentioned, there are certain damage patterns, present in archaeologically derived DNA, which are believed to indicate of its ancient origin. These patterns include short DNA fragments (exact length opinions differ between 100 bp and 500 bp), depurintaion as a main cause of fragment breakage and cytosine-to-thymine transition accumulating on the ends of DNA fragments (Grigorenko et al., 2009; Velsko et al., 2018; Ziesemer et al., 2015). It is widely considered that according to these damage patterns it is possible to distinguish ancient DNA from modern contamination, furthermore, short fragment size allows NGS library preparation without implementing initial DNA frargmentation (Briggs et al., 2007; Brotherton et al., 2007; Ginolhac et al., 2011; Jónsson et al., 2013; Key et al., 2017; Velsko et al., 2018). These parameters, however, also appear questionable under certain circumstances. It is difficult to expect precise length of ancient DNA fragments as it is highly dependent on various environmental conditions. For example, in 2006 Rogaev and colleagues has successfully retrieved mammoth mitochondrial genome fragments of length 1600 – 1700 bp from permafrost-preserved remains (Rogaev et al., 2006). Furthermore, even if we take into consideration only short fragment DNA, we still should not be expecting it to be free of environmental DNA (eDNA). In some cases, eDNA might be even more exposed to degradation factors than the DNA of bacteria that is trapped inside an archaeological sample and in this way protected from robust environment exposure. It is known that eDNA is often fragmented to the size of less then 150 bp (Pedersen et al., 2014). Likewise aDNA, its preservation is hightly dependant on the conditions of the environment it is left in. Environmental DNA in favorable conditions of cold, dry permafrost can survive for hundreds of years, whereas in the temperate water it degrades over a period of several weeks (Dejean et al., 2011; Thomsen et al., 2012; Willerslev et al., 2003). Thus, authentication of host related aDNA is a complex task and despite multiple aDNA holding protocols the risk of data bias remains high.

Apart from good laboratory practices mentioned above, specific to the field of historic DNA research, aDNA authentication nowadays is mostly dependent upon computational manipulations which operate by analyzing the damage patterns of NGS reads. Program mapDamage2.0 can be mentioned as one of the examples. This program statistically models expected deamination patterns

and further measures the estimates of damage parameters which are expected of historic DNA (Jónsson et al., 2013).

Other computational analyses in this field are mostly built towards two goals: track and distinguish sample DNA that comes from an exogenous source and distinguish sample cross-contamination, which is contamination that unintentionally comes from laboratory downstream application when working with multiple samples at a time. Example of a program that reaches for the first goal is the Bayesian model SourceTracker. This model takes into account sequenced samples together with their negative controls and estimates the origin of sample's reads together with contamination proportion (Knights et al., 2011; McGhee et al., 2020). Samples cross-contamination, on the other hand, can be assessed by usingh R package decontam. This package statistically evaluates the probability of each microorganism being contaminant by analyzing its prevalence in laboratory controls (Davis et al., 2018). These techniques, alone or combined, serve as key steps in the challenge of aDNA authentication.

1.1.5. Taxonomic assignment and data interpretation

Taxonomic assignment, that is, identifying microbial taxa present within a sample, is a standard step in metagenomics data analysis (Weyrich et al., 2017). Although numerous taxonomic classification tools are present to deal with NGS data and their number is constantly growing, accurate taxonomic assignment of metagenomics data remains a computational challenge. Main reason for this is short NGS read length which lowers the degree of assignment accuracy (Jovel et al., 2016). Ancient DNA characteristics discussed above further contribute to the difficulty of taxonomic classification of ancient microbial reads. Short reads affect assignment accuracy and cytosine-to-thymine transition may potentially cause misclassification by assigning reads to incorrect taxa or even to undefined taxa which further will result in screwed diversity estimates (Velsko et al., 2018). Nevertheless, a wide range of computational analysis tools allow to composite custom data analysis protocol, adjusting and combining tools and programs in order to reach the highest possible degree of accuracy.

In case of 16S rRNA gene sequencing, microbial community characterization usually begins with sequenced reads being clustered by two main approaches. Firstly, a reference database can be used as a template upon which to compare sequenced reads. The reads further are grouped into phylotypes depending on their level of similarity with the reference. Secondly, sequences can be formed into operational taxonomic units (OTUs) de-novo using distance matrix comparison of the datasets with no initially introduced reference (Chen et al., 2013; Jovel et al., 2016; Sun et al., 2012). First method works significantly faster, whereas second method requires notable computational

capacities. Three most widely used reference databases that are present for 16S sequencing data characterization are Greengenes, SILVA and RDP (Cole et al., 2014; DeSantis et al., 2006; Quast et al., 2013). Each database is sufficient to be used on its own, however sometimes a combination of these databases are also implemented. For example, Parallel-META 3 metagenome analysis program has a custom reference database that is created integrating Greengenes database together with SILVA and RDP in order to raise the proportion of annotated sequences (Jing et al., 2017). Furthermore, to increase the resolution of taxonomic assignment is sometimes appropriate to use a reference database that is created using bacterial taxa of specific environments only: for example, human oral cavity microbial database or human intestinal bacteria database (Forster et al., 2016; Jovel et al., 2016; Ritari et al., 2015).

Taxonomic profiling of shotgun sequencing data is also executed by a wide variety of algorithms. Regarding read clustering, one of the most popular approaches is unique clade-specific marker gene approach (such as MetaPhlAn2) where read sequences are clustered by comparing to a marker gene catalogue which is precomputed from previously sequenced bacterial genomes (Jovel et al., 2016; Truong et al., 2015). Another widely used approach is called lowest common ancestor positioning (LCA). Using this approach, pre-aligned sequences are placed on a taxonomic tree and their dissimilarity scores (bit-scores) are compared in order to assign them to a higher taxonomic level (Jovel et al., 2016). MEGAN is an example of computer program that operates implementing LCA algorithm (Huson et al., 2007). It is also possible to distinguish shotgun data metagenomics analysis approaches based on an alignment algorithm used to compare sequenced reads to a reference database. Here, the most popular algorithms would be BLAST (treating sequence as a whole entity while comparison) and k-mer based matching algorithms (dividing both reference database sequences and sequences of interest into equal length fragments – k-mers – and then matching them towards one another and using LCA principles to access their taxonomic rank. As examples of BLAST alignment algorithms can be mentioned MetaPhlAn2 and MEGAN (Huson et al., 2007; Truong et al., 2015). Kraken is an example of a program that function on a k-mer principle (Wood et al., 2019; Wood and Salzberg, 2014).

Taxonomically classified data must be further carefully interpreted with the use of statistical analysis methods. For example, Kraken – highly accurate metagenomics classification algorithm – assigns sequences to best matching location of the taxonomic tree, however it does not estimate abundance of taxonomic units. To overcome this limitation it is advisable to combine Kraken with Bracken, which stands for Bayesian Reestimation of Abundance of Species and Sequences (Lu et al., 2017). Web servers like Calypso and MicrobiomeAnalyst provide further possibilities for

comprehensive metagenomics data analysis and interpretation (Chong et al., 2020; Zakrzewski et al., 2017).

1.2. A detailed view into human oral microbiome: ancestral to modern

Oral cavity is the gateway to entire human organism. It is where food fermental pre-digestion and initial mechanical processing occurs, it is also where our immune system meets incoming nutrients and other foreign nutrient-associated elements for the first time (Abusleme and Moutsopoulos, 2017; Gaffen and Moutsopoulos, 2020; Moutsopoulos and Konkel, 2018). Taking into account strategic importance of these processes in terms of human organism's homeostasis maintenance, we can grasp the significance of human oral microbiome's function and performance. While oral cavity does harbour a great variety of microorganisms and is constantly influenced by external factors, growing evidence points to its remarkable resilience, especially comparing it to other body site's microbiome, for example that of a large intestine (Wade, 2021). Its resilience is expressed in a matter of resisting change (not to be confused with stability, which would mean returning to equilibrium after short disturbances), and is not always associated with positive health processes. For example, a pathological condition of gingivitis with condition-associated microbial community might express resilience and thus antagonize the return to the healthy state (Holling, 1973; Wade, 2021). The resilience of oral microbiota might be due to the fact that except for dietary sugars, most of oral microbial food comes from saliva and gingival crevicular fluids, thus limiting direct food influence on oral microbial communities (Beighton et al., 1986; Taylor and Preshaw, 2016). Furthermore, despite inhabiting a closely-related merged physical space of oral cavity, human oral microbial communities exhibit apparent differences between various oral cavity sites which is explained by distinctive oral niche physiochemical property differences (Aas et al., 2005). In this way, different oral tissue surfaces (mucosa, tongue, teeth etc.) exhibit various microbial communities which further obstructs historic human microbiome reconstruction using ancient dental calculus as it reflects only a fraction of individual's oral microbiome (Velsko et al., 2019). Nevertheless, despite all difficulties and possible limitations, historic human microbiome data is constantly gaining importance in the light of latest research, suggesting oral microbiome to play a crucial role not only in human oral health alone, but also in systemic health of distant body systems. Latest studies have explored the connection between oral microbiome and various systemic diseases, such as various inflammatory disorders, cardiovascular diseases, and type 2 diabetes (Lamster et al., 2008; Mercado et al., 2000; Montebugnoli et al., 2004; Reinhardt et al., 1999). The connection is also established between oral

microbiome and seemingly unrelated conditions like prostatic disease and preterm birth (Chu et al., 2018; Fang et al., 2021). These new microbiome visions contribute to holistic view of human health concept and outline new frontiers for future therapies.

Tracing back the initial composition of our ancestral oral microbial communities is highly important because it provides us the possibility to reveal the evolution of our commensal oral microbiome which is still understood poorly (Sajantila, 2013). This information, in its turn, can guide us towards the notion of what a healthy oral microbiome looks like and how are we influencing it with our lifestyle and environmental factors. At the moment three main shifts in human oral microbiome evolution can be defined. First could be agricultural revolution, which happened around 10 000 BC and marked our ancestor lifestyle transition from hunter-gatherers to farming, involving the switch to Neolithic (farming) carbohydrate-rich diets (Adler et al., 2013; Braidwood et al., 1961; Oelze et al., 2011). Early studies on ancient dental calculus have confirmed that this transition had its impact on the human oral microbiome, in particular – Neolithic dietary shift has led to the increase in disease- and dental decay-associated microorganisms (acidogenic and aciduric) (Adler et al., 2013). Furthermore, it is indicated that ancient human oral microbiome is characterized by a more significant phylogenetic diversity which hints to its increased resilience and stability in comparison to a less diverse modern oral microbiome profiles (Cadotte et al., 2012; Huttenhower et al., 2012; Lozupone et al., 2012). Next major human kind transition in terms of cultural, environmental and social factors is the Industrial Revolution – a major turning period in human history originating in Great Britain in the 18th century which is characterized by manufacturing and industrial activity becoming main social production forms (McGrath & Martin 2017). Needless to say, its influence on human diet has been tremendous. By introducing industrially processed foods, industrial period has altered core nutritional factors of human diet: glycemic load, composition of fatty acids, macro- and micronutrient composition and density, pH balance, sodium-potassium balance and fiber content (Cordain et al., 2005). Apart from direct influence on the diet, industrial era also gave humanity a new dimension of secondary human health and microbiome influence factors which originate from environmental pollution (McGrath & Martin 2017). Industrial Revolution's final stages, beginning in the mid-20th century, after the World War II, can be referred as the "Great Acceleration" – the third human lifestyle, diet, social and economic transition which shaped the world to become as we know it today (Steffen et al., 2015). The "Great Acceleration" shaped human diet in various ways: surplus food production and introduction of preservatives, high-fructose corn syrup introduction in 1970s and other food-related events. However, diet shift has not been the only major factor that shaped human oral microbiome (Popkin, 1999; Steffen et al., 2015; White, 2014). Public health moved into the next era offering society clean water, improved housing, establishing sanitation and sewage systems (Lees,

2015). Although clearly introducing remarkable benefits, public health of the Great Acceleration is also characterized by radicalized hygiene and massive antibiotic treatments. These factors are at the core of modern "hygiene hypotheses", stating that excess hygiene may be one of the reasons for modern allergy disease epidemics (Ege, 2017). Antibiotic treatments, in its turn, dramatically decrease modern microbiome diversity which makes host microbial ecosystem fragile and unstable (Iizumi et al., 2017).

Being in the beginning of microbiome research era, ancient dental calculus provides us unprecedentedly important information which can help us study our microbial heritage to better understand the connection between host and its microbiota to be able to come up with innovative non-invasive treatments for microbiome-associated diseases (Bresalier and Chapkin, 2020).

1.3. Ancient pathogens

There are many research purposes in which aDNA sequencing can be helpful. Apart from human microbiome studies aDNA gives lots of perspectives in the field of ancient pathogen studies. This relatively young scientific discipline focusing on ancient pathogen research, has so far answered several historic questions and gave support to new scientific theories. As one example, *Mycobacterium tuberculosis* co-evolution in humans is being one of most popular ancient pathogen study fields. It has been proposed that *M. tuberculosis* virulence and human resistance to infection are both likely consequences of pathogen-host historic co-evolution (Brites and Gagneux, 2015; Donoghue et al., 2004).

In many ways, Mycobacteria indeed can be considered the perfect microorganism for aDNA research. It can be found only in the infected host, this genus includes bacteria causing both tuberculosis and leprosy, and has some unambiguous advantages which facilitate the preservation of DNA in archaeological material. For example, Mycobacteria DNA is rich in guanine and cytosine, which increases DNA stability (Daffé and Draper, 1997; Donoghue et al., 2004). Also, lipid-rich thick cell wall of mycobacteria provides it additional protection against lytic enzymes and other first-stage decomposers of taphonomic cascade (Daffé and Draper, 1997; Donoghue et al., 2004; Lambert, 2002). Furthermore, given that tuberculosis and leprosy cause specific lesions in bone material, their initial detection in archaeological human remains material is implemented more easily than in case of any other pathogen, although, it cannot rely on it solely (Bos et al., 2014).

Apart from specific skeletal lesions which might indicate the possible presence of a pathogen, sometimes the burial context also hints to the presence of a specific disease as in the case of *Yersinia pestis* and historic mass graves (Bos et al., 2011; Pallen and Wren, 2007; Warinner et al., 2017). Keeping specific pathogen species in mind, targeted molecular approaches like hybridization capture protocols can be used further to identify and study pathogen's genetic information (Warinner et al., 2017). Most bacterial pathogens, however, do not exhibit any visible structural changes upon skeletal material, so their research within archaeological human remains material is most often done using nontargeted screening approaches like shotgun metagenomics (Achtman and Zhou, 2020; Neukamm et al., 2020). Metagenomics sequencing approach currently is widely used for ancient pathogen detection within various historic human tissues. As so, recent studies have successfully used shotgun metagenomics to identify ancient pathogens in human bones, dental pulp, mummified soft tissues as well as in ancient dental calculus (Achtman and Zhou, 2020; Neukamm et al., 2020; Rascovan et al., 2016).

Overall, while ancient pathogen research is confronted with obstacles both similar to other aDNA studies and unique for this specific study field, it massively contributes to our notion about infectious agent evolution by providing us phylogenetic snapshots of data which can be successfully used to reconstruct specific divergence events and widen our understanding of host-pathogen coexistence (Harkins and Stone, 2015).

2. Materials and methods

2.1. Description of samples from each publication separately

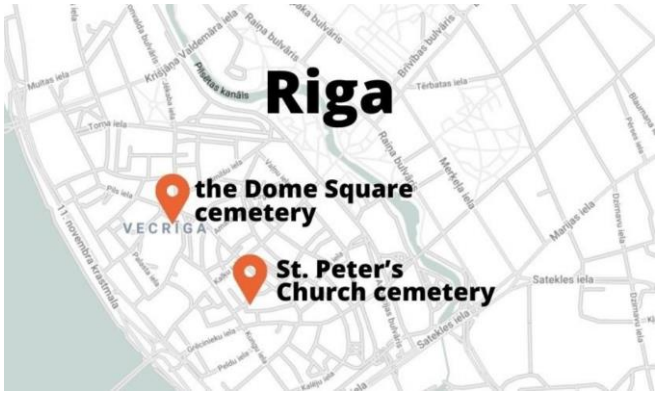
I. *"Analysis of the bacterial communities in ancient human bones and burial soil samples: Tracing the impact of environmental bacteria".*

In this study, samples of human skeletal remains from two postmedieval cemeteries in Riga, Latvia, dated 15th - 17th centuries AD were studied. Two individuals (samples K5 and K6 and soil samples Z5 and Z6, respectively) were from the Dome Square cemetery, and three individuals (samples K7, K8 and K11 and soil samples Z7, Z8 and Z11, respectively) were from St. Peter's Church cemetery (Table 1, Figure 1). Samples K5 and K6 came from partially articulated skeletons (skull was missing in both cases), samples K7 and K11 came from fully articulated skeletons, and sample K8 came from a disarticulated skeleton. Both cemeteries are located in the urban environment of the Old Riga District and are less than 400 m apart. For samples, a burial period was determined by using stratigraphy and archaeological finds (Spirģis, 2012; Tilko, 1998). The approximate age at death for individuals was assigned according to degenerative changes in the pubic symphysis and the auricular surface by standard methods described previously (Brooks and Suchey, 1990; Buckberry and Chamberlain, 2002). Soil samples were collected together with bone samples during the excavation process to evaluate the microbiome composition of the burial environment. Soil samples were collected at the burial depth from the middle section of the skeleton, i.e., between the ribcage and the pelvis, approximately 5–10 cm above the bones. All samples were packed in plastic bags and stored separately from the bone samples under the same conditions to avoid any further contamination.

Table 1. Description of samples.

Bone sample Nr	K5	K6	K7	K8	K11
Corresponding soil sample	Z5	Z6	Z7	Z8	Z11
Cemetery	Dom Square cemetery	Dom Square cemetery	St Peter's Church cemetery	St Peter's Church cemetery	St Peter's Church cemetery
Century, AD	15–16 th	16–17 th	15 th	16 th	16 th
Bone sample used	Sacrum	Vertebrae	Vertebrae	Rib	Skull
Age (years)	25–30	45–50	55–60	30–35	2–3
Sex	Male	Male	Male	Male	Child
Depth of the grave (cm)	180	190	273	173	250
Presence of debris in soil	Yes	No	No	No	No
Coffin (material)	No	No	Yes (wood)	Yes (wood)	Yes (wood)
Additional burial above	Yes (22 cm above, in wood coffin, 17th Century AD)	No	Yes (multiple)	No	Yes (multiple)
Fossil skeleton type	Partially articulated skeleton	Partially articulated skeleton	Articulated skeleton	Disarticulated skeleton	Articulated skeleton

Figure 1. Location of the two postmedieval cemeteries in Riga, Latvia.



II. *"The Postmedieval Latvian Oral Microbiome in the Context of Modern Dental Calculus and Modern Dental Plaque Microbial Profiles".*

a. *Archaeological Sample Characteristics, Collection and Burial Site Data*

During this study, 15 historic human dental calculus samples were examined, representing 6 Latvian cemeteries in 4 cities: the capital Riga (Dom Square cemetery, 56.94902, 24.10473; St. Peter's Church cemetery, 56.94752, 24.10928; St. Gertrude's Church cemetery, 56.95799, 24.12172), Cesis (St. John's Church cemetery, 57.31213, 25.27168), Kuldiga (Church of the Holy Trinity, Roman Catholic Church, 56.96765, 21.96943) and Jelgava (St. Trinity's Church, 56.65239, 23.72897), (Table 2, Figure 2,3). All samples were dated to 16–17th century AD. Most individuals were in their young adulthood to middle age (20–60 years of age) at the time of death. Sample ZA_7C was suspected to represent a teenager (14–15 years of age). Prior to dental calculus sample collection, archaeological skeletons were inspected for the presence of any disease-specific leisure signs. Mouth cavities were inspected for the presence of oral disease lesions (Ogden, 2007). Four archaeological skeletons were found to exhibit tooth decay signs, 10 skeletons exhibited specific and nonspecific bone lesions, and for four skeletons, no signs of diseases were observable (Table 1). The teeth and the alveolar bone appeared to be macroscopically sound without traces of periodontal disease. To determine the burial period of the samples, the stratigraphy method was used together with the evaluation of archaeological finds (Spirģis, Tilko). The approximate age at death was determined by evaluating degenerative changes in the pubic symphysis and using the auricular surface standard method (Brooks and Suchey, 1990; Buckberry and Chamberlain, 2002).

Random soil samples were collected together with bone samples during the excavation process to evaluate the microbiome composition of the burial environment. Burial soil samples were collected from two cemeteries in Riga, Latvia: Dome Square cemetery (samples Z5 and Z6_sk) and St. Peter's Church cemetery (samples Z7 and Z7_sk). Soil samples were

collected at the burial depth from the middle section of the skeleton, i.e., between the ribcage and the pelvis, approximately 5–10 cm above the bones. All samples were packed in plastic bags and stored separately from the bone samples under the same conditions to avoid any further contamination.

b. Modern Dental Calculus and Modern Dental Plaque Sample Collection

Modern dental supragingival calculus samples (n = 4) were collected at the Institute of Stomatology (Riga Stradins University, Riga, Latvia) by a professional dentist during the process of routine dental cleaning (Table 2). Dental calculus samples were collected with the use of a dental scaler. The exact location of the sample taken was selected arbitrarily by the dentist. Modern dental supragingival plaque samples (n = 20) were collected by rubbing a cotton swab over the supragingival sites of the teeth. All samples were collected with patient consent, and the study was reviewed and approved by Riga Stradins University Research Ethics Committee, decision no. 6-3/4/5 (25 April 2019).

Table 2. Description of samples.

Sample ID	City	Cemetery	Century, AD	Year of Excavation	Age (Years)	Sex	Lifestyle	Possible Diseases	Tooth Type
ZA_1C	Riga	Dom Square	16–17th	1986	30–40	Male	Town/city, middle classes	Inflammation of the clavicle	Molar
ZA_3C	Riga	Dom Square	16–17th	1986	45–50	Female	Town/city, middle classes	Caries	Incisor
ZA_4C	Riga	St. Peter's Church	16–17th	2004	30–35	Male	Town/city	-	Incisor
ZA_5C	Riga	St. Peter's Church	16–17th	2004	25–30	Male	Town/city	Fractured rib and left arm's fracture	Incisor
ZA_6C	Riga	St. Peter's Church	16–17th	2004	20–25	Male	Town/city	Non-specific inflammation of the lower leg bone surface	Premolar
ZA_7C	Riga	St. Gertrude's Church	16–17th	2006	14–15	Unknown	Countryside, commuter town	-	Incisor
ZA_8C	Riga	St. Gertrude's Church	17th	2006	45–50	Male	Countryside, commuter town	Deforming arthrosis (joints)	Incisor
ZA_9C	Riga	St. Gertrude's Church	16–17th	2006	25–30	Male	Countryside, commuter town	Vertebral fracture with local inflammation	Molar
ZA_10C	Riga	St. Gertrude's Church	16–17th	2006	55–60	Male	Countryside, commuter town	Caries, tooth root abscess, arthritis in joints	Molar
ZA_11C	Cesis	St. John's Church	17th	2015	35–40	Male	Town/city, aristocracy	Multiple tooth decay, toe osteomyelitis	Incisor
ZA_12C	Cesis	St. John's Church	17th	2015	35–40	Female	Town/city, aristocracy	Non-specific inflammatory process on the surface of the leg bones	Premolar
ZA_20C	Kuldiga	Church of the Holy Trinity, Roman Catholic Church	16–17th	2015	45–50	Female	Town/city, lower classes	-	Molar
ZA_22C	Kuldiga	Church of the Holy Trinity, Roman Catholic Church	16–17th	2015	30–35	Female	Town/city, lower classes	-	Incisor
ZA_29C	Jelgava	St. Trinity's Church	16–17th	2009	35–40	Female	Town/city, aristocracy	Caries, arthritis in joints, non-specific inflammation	Premolar
ZA_C22	Riga	St. Gertrude's Church	17th	2006	40–50	Unknown	Countryside, commuter town	Deforming arthrosis, pelvic joint	Incisor

Figure 2. Location of cemeteries.

Figure 3. Examples of samples.

III. *"Exploring DNA preservation and taxonomic diversity in postmedieval human tooth samples in Latvia".*

Archaeological human tooth samples, dated between the 15th and 17th centuries AD, were collected from three cemeteries in Riga, Latvia: St. Gertrude's Church cemetery (samples T2,

T3, T9); the Dome Square cemetery (sample TZA3), and St. Peter's Church cemetery (sample TZA4) (Table 3, Figure 4,5). St. Peter's Church cemetery and the Dome Square cemetery are located in the Old Riga District, whereas St. Gertrude's Church cemetery is located outside the Old Riga medieval city wall and has been associated with St. Gertrude's medieval village. Prior to tooth sample collection, archaeological skeletons were inspected for the presence of any disease-specific leisure signs. Special attention was given to tuberculosis and leprosy specific bone changes, as these diseases are known to exhibit very distinct lesions on skeletons (Lewis et al., 1995; Pálfi et al., 2015). Mouth cavities were inspected for the presence of oral disease lesions and dental calculus. Four skeletons exhibited specific and nonspecific bone lesions, and for one skeleton, no signs of diseases were observable (Table 1). The burial period of the samples was determined by the stratigraphy method, which was used together with the evaluation of archaeological finds (Spirģis, 2012; Tilko, 1998). The approximate age at death was determined by evaluating degenerative changes in the pubic symphysis and using the auricular surface standard method (Brooks and Suchey, 1990; Buckberry and Chamberlain, 2002). Sex was estimated by an experienced anthropologist using pelvic and cranial criteria (Phenice, 1969) (Ascádi and Nemeskéri, 1970; Phenice, 1969).

Table 3. Description of samples.

Sample ID	T2	T3	T9	TZA3	TZA4
Cemetery	St. Gertrude's Church	St. Gertrude's Church	St. Gertrude's Church	Dome Square	St. Peter's Church
Century, AD	17th	17th	17th	16-17th	15-16th
Year of excavation	2006	2006	2006	1986	2004
Depth of the grave/sample taken (cm)	320	320	280	150	270
Additional burial above	yes	yes	yes	yes	yes
Fossil skeleton type	Disarticulated skeleton	Articulated skeleton	Articulated skeleton	Articulated skeleton	Articulated skeleton
Sample type	Tooth (molar)	Tooth (premolar)	Tooth (molar)	Tooth (incisor)	Tooth (incisor)
Dental calculus	Yes	No	No	Yes	Yes
Possible diseases	Fusion of the spine, scoliosis, tooth root abscess	Fusion of the spine with local inflammation	Fusion of the spine	Arthritis, periostitis	–
Age (years)	30–35	20–25	16–18	45–50	30–35
Sex	female	male	unknown	female	male
DNA concentration (ng/µl)	1.5	0.91	1.7	3.5	3.8
Mean DNA fragment length (bp)	2705	5058	4449	549	432
Median DNA fragment length (bp)	3200	7000	5500	4000	1600

Figure 4. Location of cemeteries.

Figure 5. Excavations of St. Gertrude's Church cemetery (Image taken from Rudovica et al., 2011).

2.2. DNA isolation

I. *"Analysis of the bacterial communities in ancient human bones and burial soil samples: Tracing the impact of environmental bacteria".*

In this study, the guidelines proposed for aDNA research were followed to minimize the risk of contamination (Fulton and Shapiro, 2019). DNA isolation was performed in a laboratory fully dedicated to aDNA research, and a "one-way" rule of movement was maintained. The aDNA facilities were strictly isolated from the locations where PCRs were performed and consisted of physically separated areas for sample preparation, DNA extraction and PCR set-up. All standard precautions such as the use of dedicated protective clothing and disposables were taken (Donoghue, 2007). Personal wear included disposable full-body suits, surgical facemasks, plastic see-through visors, and two layers of gloves. The facilities were illuminated with ultra-violet (UV) light for 30 min prior to each experiment, and the floors and surfaces were cleaned weekly with a 5% sodium hypochlorite (NaClO) solution. DNA extractions and amplification preparations were performed in a room separate from sample preparation and were completed in still-air cabinets that were cleaned with 5% NaClO and UV illuminated. To eliminate possible contamination with modern DNA, the surfaces of the bones were cleaned by immersing in 5% NaOCl and rinsing with nuclease-free water. The bones were irradiated for 2 h with UV light with 6 J/cm2 at 254 nm on each side before processing and leaving to dry overnight at room temperature. A portion of the bones was cut out with a cutting disc for analysis and pulverized using a CryoMill (RETSCH, Germany). All instruments and surfaces involved in the process were treated with NaOCl and UV light prior to and after each procedure for decontamination. Only one bone at a time was processed. DNA was extracted from 2 g of bone powder using the method described elsewhere (Keyser-Tracqui and Ludes, 2005). Purification and concentration of DNA samples were performed using the Genomic DNA Clean & Concentrator Kit (Zymo Research, United States) following the manufacturer's instructions. DNA concentrations were estimated using a Qubit dsDNA HS Assay Kit (Life Technologies, United States). To relate the results and compare them fully, the DNA extractions from the corresponding soil samples were performed using the same reagents and kits as for the bone samples. To control laboratory contamination, blank samples were included in each experiment of DNA isolation and processed simultaneously with the corresponding samples.

II. *"The Postmedieval Latvian Oral Microbiome in the Context of Modern Dental Calculus and Modern Dental Plaque Microbial Profiles".*

Ancient DNA (aDNA) handling in laboratory conditions requires special care and precautions to eliminate all possible contamination from modern DNA sources. This study strictly

followed specific guidelines developed exclusively for aDNA research (Donoghue, 2007; Fulton and Shapiro, 2019). aDNA handling protocols consist of actions prescribing facility preparation prior to aDNA handling, instrument treatment, facility worker equipment and archaeological material processing. Ancient DNA facility preparation required regular ultraviolet (UV) light irradiation and weekly surface and floor cleaning actions with 5% sodium hypochloride (NaClO) solution. All instruments involved in aDNA procedures were treated similarly by washing with 5% NaOCl solution and irradiating with UV light before and after each procedure. Archaeological samples were processed one at a time. The aDNA facility consisted of three strictly separated chambers, each serving its defined purpose. Archaeological material preprocessing and pulverized sample incubation occurred in one facility chamber, and another two chambers were devoted to aDNA isolation and library preparation. The fourth chamber (buffer zone) separated the aDNA facility from the rest of the laboratory. Archaeological tooth samples carrying the desired dental calculus remains were first immersed in 5% NaOCl solution, rinsed with nuclease-free water and irradiated for 2 h with UV light (6 J/cm2 at 254 nm). Samples were then left to dry overnight at room temperature. The next day, dental calculus was cautiously removed from the surface of the teeth with a scalpel and was ground inside a tube with a sterile microbiological stick. Laboratory blank samples (BC and BC_sk) were processed simultaneously with archaeological samples. DNA extraction was performed as described previously (Kazarina et al., 2019; Keyser-Tracqui and Ludes, 2005). Burial soil and modern dental calculus samples were processed in another facility to avoid possible cross-contamination with aDNA samples. Burial soil samples that were chosen to represent the soil microbiome of the burial environment of St. Peter's Church cemetery (samples Z5 and Z6_sk) and St. Gertrude's Church cemetery (samples Z7 and Z7_sk) underwent the same procedures of DNA extraction and purification as the aDNA samples. Modern dental calculus samples were washed with 5% NaOCl solution and were then rinsed with nuclease-free water. Furthermore, together with the modern dental plaque samples, modern dental calculus samples underwent a DNA extraction process. All resultant DNA samples were inspected with a Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA) to estimate the resultant DNA concentration (Table S1, Supplementary Materials).

III. *"Exploring DNA preservation and taxonomic diversity in postmedieval human tooth samples in Latvia".*

During the work with archaeological samples, special care was taken to avoid DNA contamination. To fulfill the safety criteria of aDNA handling and minimize the risk of

contamination, specific guidelines developed for aDNA research were followed (Donoghue, 2007; Fulton and Shapiro, 2019). All the surfaces and instruments were cleaned and prepared for aDNA experiments according to rigorous protocols, including cleaning with 5% NaOCl solution and irradiation with UV light (Kazarina et al., 2019). Facility workers were equipped with disposable surgical facemasks, full-body suits and gloves, and only one tooth sample at a time was processed to avoid cross-contamination.

Archaeological material was prepared for aDNA isolation in a specially designated separated area of aDNA research facilities. A well-preserved tooth was sampled for each individual. The surfaces of the teeth were abraded with single-use scalpel equipment to remove the calculus when present, and DNA extraction was performed as described previously (Kazarina et al., 2019; Keyser-Tracqui and Ludes, 2005). Briefly, tooth samples were rinsed in bleach (5% solution for 30 sec), rinsed with nuclease-free water and exposed to UV light for 30 min on each side. Samples were then left to dry overnight at room temperature. The next day, whole tooth samples were reduced to fine powder using a CryoMill (RETSCH, Germany). Sample decalcification was performed by incubation of 1 g of powder in extraction buffer (5 mM ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 8.0), 0.3 M sodium acetate, and 1 mg proteinase-K/mL) overnight at 50°C with continuous vertical rotation. DNA was separated from cellular debris by phenol/chloroform/isoamyl alcohol (25/24/1, v/v) extraction. Purification and concentration of DNA samples were performed using the Genomic DNA Clean & Concentrator Kit (Zymo Research, United States) following the manufacturer's instructions. The DNA concentration was estimated using a Qubit dsDNA HS Assay Kit (Life Technologies, United States), and the assessment of DNA quality and fragment length was performed using an Agilent High Sensitivity DNA Kit (Agilent Technologies, United States). To control laboratory contamination, DNA isolation procedures were accompanied by corresponding blank samples, which were also treated equally and underwent the same DNA isolation steps using the same DNA purification and concentration reagents.

2.3. Library preparation and sequencing

I. *"Analysis of the bacterial communities in ancient human bones and burial soil samples: Tracing the impact of environmental bacteria".*

The amplicon library was prepared using the Ion 16S™ Metagenomics Kit (Life Technologies, United States) following the manufacturer's instructions. Briefly, bacterial 16S rRNA genes were amplified with two sets of primers provided: primer set V2-4-8 and primer

set V3-6, 7-9. Prior to the sequencing process, all samples were examined for fragment size distribution, library quality and concentration using an Agilent High Sensitivity DNA Kit and Bioanalyser 2100 instrument (Agilent Technologies, United States). Metagenome sequencing was performed on an Ion Torrent (PGM) Platform 318 v2 chip according to the manufacturer's instructions (Life Technologies, United States). All individual sequence reads underwent filtering within the PGM software to remove polyclonal and low-quality sequences. Barcodes and PGM adapters were also automatically trimmed. The resulting data were exported in the form of Bam files.

II. *"The Postmedieval Latvian Oral Microbiome in the Context of Modern Dental Calculus and Modern Dental Plaque Microbial Profiles".*

Before library preparation, modern dental calculus, modern dental plaque DNA samples, and burial soil DNA samples Z6_sk (St. Peter's Church cemetery) and Z7_sk (St. Gertrude's Church cemetery), underwent an additional DNA fragmentation step using the Ion ShearTM Plus Reagent Kit (Ion Torrent™, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The fragmentation conditions were selected according to the desired fragment size (150–250 bp). For the historic dental calculus samples and for burial soil samples Z5 (St. Peter's Church cemetery) and Z7 (St. Gertrude's Church cemetery), the DNA fragmentation step was omitted to ensure the capture of short DNA fragments that are believed to represent aDNA (Dabney et al., 2013; Key et al., 2017). DNA samples underwent a size-selection procedure to remove DNA fragments larger than 250 bp using NucleoMag®NGS Clean-up and Size Select magnetic beads (Macherey-Nagel), and for library preparation, an Ion Plus Fragment Library Kit (Ion Torrent™) was used. To evaluate sample contamination from laboratory sources, two laboratory control samples were processed with the historical DNA samples and sequenced: aDNA extraction blank sample (BC) and aDNA extraction blank sample, which also underwent a DNA fragmentation step (BC_sk). Preparation of all sequencing libraries followed the same steps regardless of sample origin. Specific barcodes were ligated, and libraries underwent amplification and quality assessment using an Agilent High Sensitivity DNA Kit and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. Sequencing was performed on an Ion ProtonTM System (Ion Torrent™, Thermo Fisher Scientific, Waltham, MA, USA). All raw historic calculus DNA sequencing data are publicly available at the ENA under accession PRJEB40382.

III. *"Exploring DNA preservation and taxonomic diversity in postmedieval human tooth samples in Latvia".*

Metagenomics sequencing libraries were prepared using the Ion Plus Fragment Library Kit (Ion Torrent™, Thermo Fisher Scientific, USA) following the manufacturer's instructions. Each DNA sample was divided into two aliquots of 10 µl each, which were further used to prepare two types of libraries: non-fragmented and fragmented libraries. For non-fragmented libraries, with the intention of keeping only short DNA fragments (<350 bp), aliquots underwent size selection using NucleoMag® NGS Clean-up and Size Select magnetic beads (Macherey-Nagel, USA). In this study, these libraries were named "Short DNA library". For fragmented libraries, with the intention of analyzing the total DNA in the samples, including long DNA fragments, prior to specific barcode attachment, aliquots underwent enzymatic fragmentation with an Ion ShearTM Plus Reagent Kit (Ion Torrent™, Thermo Fisher Scientific, USA) following the manufacturer's instructions. In this study, these libraries were named "Total DNA library". Further library preparation steps were identical for both library types and, according to the manufacturer's instructions, involved the following steps: specific barcode ligation, library amplification and assessment of library quality on the Bioanalyzer 2100 instrument with an Agilent High Sensitivity DNA Kit (Agilent Technologies, USA). Sequencing was performed on an Ion Proton™ System (Thermo Fisher Scientific, USA).

2.4. Sequencing data analysis

I. *"Analysis of the bacterial communities in ancient human bones and burial soil samples: Tracing the impact of environmental bacteria".*

The resulting metagenomic data were analyzed with a variety of computational methods. The Galaxy public server was used to remove two overrepresented remaining adapter sequences and select highquality data from the remaining sequences; reads with a quality PHRES score < 20 were excluded (Goecks et al., 2010; Kosakovsky Pond et al., 2009). Taxonomic and functional profiling and representation were performed using Parallel-META 3 (Jing et al., 2017; Su et al., 2012). The relative abundance of bacterial taxons was calculated using the Parallel-META program as a proportion of reads mapped to a specific bacterial phylum (Jing et al., 2017). Microbiota diversity within samples (alpha diversity) was assessed by the Shannon diversity index, which evaluates the richness and evenness of taxa. Significant differences in microbial community composition between groups of samples (beta diversity) were calculated by ANOSIM (Bray-Curtis distance method) and illustrated by principal

component analysis (PCA). Statistical analyses were executed and visualized using Calypso software (cgenome.net/calypso/) (Zakrzewski et al., 2017).

II. *"The Postmedieval Latvian Oral Microbiome in the Context of Modern Dental Calculus and Modern Dental Plaque Microbial Profiles".*

Sequencing data preprocessing on the local Ion Torrent Proton server included initial quality control steps as well as data assignment to each individual sample. Barcodes and sequencing adapters, together with polyclonal and low-quality sequences, were filtered by Proton software during the first post-sequencing data handling step. The resultant data were exported for further manipulations in the form of BAM files. The resulting exported BAM files were initially quality-processed using the Galaxy public server (Goecks et al., 2010; Kosakovsky Pond et al., 2009). Briefly, overrepresented sequences were removed, and reads with quality PHRED scores < 20 were excluded. Sequencing data taxonomic assignment was performed with Kraken2 v2.0.7 using the standard Kraken2 database (Wood et al., 2019). Bracken (Bayesian Reestimation of Abundance with Kraken) was used to compute the abundance of species in DNA sequences from a metagenomics sample (Lu et al., 2017).

Pavian R application (Breitwieser and Salzberg, 2020) was used to further manipulate Kraken/Bracken taxonomy report files, generate quality assignment and prepare data for statistical analysis and representation, which was done using the MicrobiomeAnalyst (https://www.microbiomeanalyst. ca/ (accessed on 23 December 2020)) web application (Chong et al., 2020; Dhariwal et al., 2017).

The Bayesian analysis-based program SourceTracker (Knights et al., 2011) was used to evaluate the possible source of predominant microbial signatures of historic dental calculus samples to enable oral microbiome preservation assessment and to track the influence of exogenous microbial contamination. All source files, which were included in the pipeline, were defined from these study samples. There were five sources defined: modern supragingival calculus, modern supragingival plaque, two aDNA extraction blanks and burial soil. The open-source R package decontam (https://github.com/benjjneb/decontam (accessed on 23 December 2020)) (Davis et al., 2018) was used for the identification and removal of laboratory contaminants in metagenomics data. Low-abundance species were removed by applying a hard cutoff (0.001% abundance).

The authenticity of historic specimen microbiome data was confirmed using DNA damage patterns. This method is based on the hypothesis that DNA deamination rates increase over time (Dabney et al., 2013), and characteristic features of damaged DNA patterns may confirm the origin of the DNA. For this purpose, read files of historic dental calculus samples were

analyzed using MALT 0.5.0 (Herbig et al., 2016) (https://software-ab.informatik.uni-tuebingen.de/ download/malt/welcome.html (accessed on 23 December 2020)), using all complete bacterial genomes available from NCBI Assembly in August 2020 as a reference. Bacterial reads were extracted using SAMtools, mapped to the reference genome of the prevalent oral microorganism Olsenella sp. oral taxon 807 and Actinomyces sp. oral taxon 414, and DNA deamination rates were calculated using mapDamage (Jónsson et al., 2013) by the EAGER pipeline (Peltzer et al., 2016).

Intergroup differences in alpha diversity were assessed by the Shannon diversity index. Beta diversity was tested by permutational multivariate analysis of variance (PERMANOVA), a nonparametric multivariate statistical test (Anderson, 2001) presented by principal coordinates analysis (PCoA). Hierarchical clustering was visualized by dendrogram and heatmap analysis using the Bray–Curtis similarity index and Ward clustering algorithm. Intergroup differences at the species level were analyzed using the Kruskal–Wallis test and the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011) with default settings on the MicrobiomeAnalyst website; the threshold on the logarithmic LDA score for discriminative features was set to 2.0, and the false discovery rate (FDR)-adjusted p value cutoff was set to 0.05.

III. *"Exploring DNA preservation and taxonomic diversity in postmedieval human tooth samples in Latvia".*

Sequencing data were preprocessed on the local Ion Torrent Proton server, assigning data to each sample and removing barcodes and adapters. The resulting data were exported from the server in the format of BAM files, which further underwent a quality control workflow and removal of two overrepresented remaining adapter sequences using analysis tools on the Galaxy public server (Goecks et al., 2010, Kosakovsky Pond et al., 2009). Sequences that underwent quality filtering and reads with quality PHRES scores <20 were excluded from further manipulations. Kraken2 v2.0.7 with the use of the standard Kraken2 database (Wood and Salzberg, 2014) was used to assign taxonomic labels to the resultant metagenomic DNA sequences. Kraken taxonomy report files were further manipulated with the use of the Pavian web application – taxonomy quality assignment was generated (Breitwieser et al., 2020). Contamination control was performed using the R package "Decontam" (Davis et al., 2018, Salter et al., 2014).

Statistical analyses were performed and visualized using the MicrobiomeAnalyst public server (https://www.microbiomeanalyst.ca/) (Chong et al., 2020, Dhariwal et al., 2017) and Calypso public server (https://cgenome.net/calypso/) (Zakrzewski et al., 2017). Community

alpha diversity was estimated by the Shannon diversity index, which evaluates both the richness and evenness of taxa within the samples. Differences in beta diversity between sample clusters were calculated using analysis of similarities (ANOSIM) and a Bray-Curtis dissimilarity matrix and displayed by principal component analysis (PCA).

To identify the presence of human DNA, sequencing reads obtained from tooth samples were processed in silico with the Efficient Ancient Genome Reconstruction pipeline (EAGER, v.1.92) (Peltzer et al., 2016). De-multiplexed, adapter-clipped reads were aligned to a human genome reference (GRCh38, GCA_000001405.28) with BWA (Li and Durbin, 2009). DamageProfiler was used to characterize DNA damage (Neukamm et al., 2020). For the characterization of genomic data, the PALEOMIX pipeline was used (http://geogenetics.ku.dk/publications/paleomix) (Schubert et al., 2014).

Preservation of oral microbial DNA in historic tooth samples was assessed. The metagenomics sequencing read files of historic tooth samples were analyzed using MALT 0.5.0 (Herbig et al., 2016) (https://software-ab.informatik.uni-tuebingen.de/download/malt/welcome.html), using all complete bacterial genomes available from NCBI Assembly in August 2020 as a reference. Bacterial reads were extracted using SAMtools, mapped to the reference genomes of the oral microorganisms Olsenella sp. oral taxon 807 and Streptococcus sanguinis, and DNA deamination rates were calculated using MapDamage (Jónsson et al., 2013) within the EAGER pipeline.

3. Results

I. Analysis of the bacterial communities in ancient human bones and burial soil samples: Tracing the impact of environmental bacteria.

Highlights:

Part of the results from this publication was used in these theses.

- The microbiome of archaeological bone and corresponding soil samples was studied.
- Differences in beta-diversity were observed at both the phyla and genus levels.
- Firmicutes abundance was significantly different between bone and soil samples.
- Differences in alpha- and beta-diversities were observed for Firmicutes genera.

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Analysis of the bacterial communities in ancient human bones and burial soil samples: Tracing the impact of environmental bacteria

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ABSTRACT

In our attempts to reveal the hidden fragments of the history of the natural world, ancient DNA (aDNA) is the precious missing key that allows us to discover hidden truths about ourselves and the world around us. Not only does aDNA encrypt genetic data from a particular individual, it also carries information about the microbial communities that were present in the individual. However, the process of such data mining has many intrinsic challenges. One of the main challenges in aDNA research is the contamination of archaeological material with environmental bacteria from the surrounding soil and postmortem microbial sources.

The goal of this study was to identify the microbial communities in human archaeological bone samples dated 15th - 17th century AD and to compare the microbiome patterns with the corresponding soil samples of the burial environment. Samples were analysed by 16S rRNA-based profiling of bacterial communities using Ion Torrent technology. The results showed that the most represented phyla in the bone samples were Firmicutes followed by Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Nitrospirae, Planctomycetes, Gemmatimonadetes and Bacteroidetes.

All identified microbial taxa of the bone samples coincided with the corresponding soil samples, indicating significant infiltration and contamination of archaeological remains with microbial species of the burial environment. However, differences in microbial community composition between the bone and soil samples were observed at both the phylum and genus levels, as indicated by statistically significant beta-diversity analysis results. A deeper investigation of the Firmicutes phylum showed significant differences between the bone and soil samples by alpha- and beta-diversity analyses. Several genera belonging to Firmicutes were significantly more abundant in the soil samples than in the bone samples and vice versa.

In conclusion, the analysis of the microbiome profiles of archaeological bone and corresponding soil samples revealed significant diversity in microbial compositions. It appeared that some bacteria may infiltrate the bone matter through the process of tissue decomposition and remain trapped inside for a longer period of time. Archaeological human bone samples might provide significant data on the investigation of ancient human microbiomes; however, environmental bacteria from the surrounding soil must be considered an important contamination factor.

1. Introduction

Ancient DNA (aDNA) analysis is a relatively young and, in many ways, unique and powerful tool that can lead to fascinating discoveries in the field of natural history – discoveries that could not have been made using other research methods. In particular, aDNA research has provided the notion of some key aspects of our demographic history and made it possible to discover different features of our evolutionary history, allowing us to explore our genetic relationship with our extinct hominin relatives (Green et al., 2010; Meyer et al., 2012). Moreover, the capabilities of aDNA research expand even further as aDNA also carries information about microbial communities that were present in the organism of the individual at the time of death. This research sheds light on problems related to ancient human diseases, the evolution of ancient pathogens, and, in general, ancient human nutrition and life conditions (Bos et al., 2011; Warinner et al., 2014, 2015). This information is valuable as a historical record, and currently, it has enormous potential to contribute to the goals of modern healthcare.

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In addition to frozen or mummified human remains, coprolites and dental calculus can be considered a trustworthy snapshot of a particular organism's ancient microbiome (Warinner et al., 2015; Mann et al., 2018). Ancient bone samples might be helpful in the investigation of the ancient human microbiome because of the idea that human tissue morphology rapidly changes after the end of life, and bacteria from different tissues may infiltrate bones shortly after an individual's death and remain trapped inside (Bell et al., 1996; Morris et al., 2006). However, one of the main challenges in aDNA research is the contamination of archaeological material with environmental bacteria from surrounding soil and postmortem microbial sources. Due to soft tissue decomposition, the microbiome of a particular organism changes dramatically soon after death (Saegeman et al., 2009; Heimesaat et al., 2012). Thus, the profile of the ancient microbiome could be altered because of postmortem microbial overgrowth and shifted towards microbial species that are most abundant during the stages of decomposition (Santiago-Rodriguez et al., 2015). In addition, fragmented aDNA usually makes up less than 1% of the total genetic material extracted from a sample, and the majority of DNA usually stems from the environment (Spyrou et al., 2019).

This fact is especially important in ancient pathogen research. As an example, the presence of environmental bacteria in human archaeological remains has been highlighted as a complication in the study of ancient tuberculosis, as it was shown that environmental mycobacteria, as well as bacteria of related genera, were present in most if not all of the archaeological remains studied (Müller et al., 2016). Thus, the assessment of the taphonomic processes of the bone material and the contamination of aDNA with modern soil bacteria is crucial for the legitimate evaluation of ancient microbiomes.

The goal of this study was to identify microbial communities in human archaeological bone remains from Latvia dated 15th - 17th century AD and the corresponding soil samples and to evaluate the impact of environmental bacterial contamination that comes from the surrounding soil.

2. Materials and methods

2.1. Archaeological sites and morphological analysis of human skeletal remains

In this study, samples of human skeletal remains from two medieval cemeteries in Riga, Latvia, dated 15th - 17th centuries AD were studied. Two individuals (samples K5 and K6 and soil samples Z5 and Z6, respectively) were from the Dome Square cemetery, and three individuals (samples K7, K8 and K11 and soil samples Z7, Z8 and Z11, respectively) were from St. Peter's Church cemetery (Table 1). Samples K5 and K6 came from partially articulated skeletons (skull was missing in both cases), samples K7 and K11 came from fully articulated skeletons, and sample K8 came from a disarticulated skeleton.

Both cemeteries are located in the urban environment of the Old Riga District and are less than 400 m apart. For samples, a burial period was determined by using stratigraphy and archaeological finds (Spirģis, 2012; Tilko, 1998). The approximate age at death for individuals was assigned according to degenerative changes in the pubic symphysis and the auricular surface by standard methods described previously (Brooks and Suchey, 1990; Buckberry and Chamberlain, 2002).

Soil samples were collected together with bone samples during the excavation process to evaluate the microbiome composition of the burial environment. Soil samples were collected at the burial depth from the middle section of the skeleton, i.e., between the ribcage and the pelvis, approximately 5–10 cm above the bones. All samples were packed in plastic bags and stored separately from the bone samples under the same conditions to avoid any further contamination.

2.2. DNA isolation

In this study, the guidelines proposed for aDNA research were followed to minimize the risk of contamination (Fulton and Shapiro, 2019). DNA isolation was performed in a laboratory fully dedicated to aDNA research, and a "one-way" rule of movement was maintained. The aDNA facilities were strictly isolated from the locations where PCRs were performed and consisted of physically separated areas for sample preparation, DNA extraction and PCR set-up. All standard precautions such as the use of dedicated protective clothing and disposables were taken (Donoghue, 2008). Personal wear included disposable full-body suits, surgical facemasks, plastic see-through visors, and two layers of gloves. The facilities were illuminated with ultra-violet (UV) light for 30 min prior to each experiment, and the floors and surfaces were cleaned weekly with a 5% sodium hypochlorite (NaClO) solution. DNA extractions and amplification preparations were performed in a room separate from sample preparation and were completed in still-air cabinets that were cleaned with 5% NaClO and UV illuminated. To eliminate possible contamination with modern DNA, the surfaces of the bones were cleaned by immersing in 5% NaOCl and rinsing with nuclease-free water. The bones were irradiated for 2 h with UV light with 6 J/cm2 at 254 nm on each side before processing and leaving to dry overnight at room temperature. A portion of the bones was cut out with a cutting disc for analysis and pulverized using a CryoMill (RETSCH, Germany). All instruments and surfaces involved in the process were treated with NaOCl and UV light prior to and after each procedure for decontamination. Only one bone at a time was processed.

DNA was extracted from 2 g of bone powder using the method described elsewhere (Keyser-Tracqui and Ludes, 2005). Purification and concentration of DNA samples were performed using the Genomic DNA Clean & Concentrator Kit (Zymo Research, United States) following the manufacturer's instructions. DNA concentrations were estimated using a Qubit dsDNA HS Assay Kit (Life Technologies, United States). To relate the results and compare them fully, the DNA extractions from the

Table 1
Characteristics of samples.

Bone sample Nr	K5	K6	K7	K8	K11
Corresponding soil sample	Z5	Z6	Z7	Z8	Z11
Cemetery	Dom Square cemetery	Dom Square cemetery	St Peter's Church cemetery	St Peter's Church cemetery	St Peter's Church cemetery
Century, AD	15–16 th	16–17 th	15 th	16 th	16 th
Bone sample used	Sacrum	Vertebrae	Vertebrae	Rib	Skull
Age (years)	25–30	45–50	55–60	30–35	2–3
Sex	Male	Male	Male	Male	Child
Depth of the grave (cm)	180	190	273	173	250
Presence of debris in soil	Yes	No	No	No	No
Coffin (material)	No	No	Yes (wood)	Yes (wood)	Yes (wood)
Additional burial above	Yes (22 cm above, in wood coffin, 17th Century AD)	No	Yes (multiple)	No	Yes (multiple)
Fossil skeleton type	Partially articulated skeleton	Partially articulated skeleton	Articulated skeleton	Disarticulated skeleton	Articulated skeleton

corresponding soil samples were performed using the same reagents and kits as for the bone samples. To control laboratory contamination, blank samples were included in each experiment of DNA isolation and processed simultaneously with the corresponding samples.

2.3. Library preparation and sequencing

The amplicon library was prepared using the Ion 16S™ Metagenomics Kit (Life Technologies, United States) following the manufacturer's instructions. Briefly, bacterial 16S rRNA genes were amplified with two sets of primers provided: primer set V2-4-8 and primer set V3-6, 7-9. Prior to the sequencing process, all samples were examined for fragment size distribution, library quality and concentration using an Agilent High Sensitivity DNA Kit and Bioanalyser 2100 instrument (Agilent Technologies, United States). Metagenome sequencing was performed on an Ion Torrent (PGM) Platform 318 v2 chip according to the manufacturer's instructions (Life Technologies, United States). All individual sequence reads underwent filtering within the PGM software to remove polyclonal and low-quality sequences. Barcodes and PGM adapters were also automatically trimmed. The resulting data were exported in the form of Bam files.

2.4. Metagenome analysis

The resulting metagenomic data were analysed with a variety of computational methods. The Galaxy public server was used to remove two overrepresented remaining adapter sequences and select high-quality data from the remaining sequences; reads with a quality PHRES score < 20 were excluded (Goecks et al., 2010; Kosakovsky Pond et al., 2009). Taxonomic and functional profiling and representation were performed using Parallel-META 3 (Jing et al., 2017; Su et al., 2012). The relative abundance of bacterial taxons was calculated using the Parallel-META program as a proportion of reads mapped to a specific bacterial phylum (Jing et al., 2017).

Microbiota diversity within samples (alpha diversity) was assessed by the Shannon diversity index, which evaluates the richness and evenness of taxa. Significant differences in microbial community composition between groups of samples (beta diversity) were calculated by ANOSIM (Bray-Curtis distance method) and illustrated by principal component analysis (PCA). Statistical analyses were executed and visualized using Calypso software (cgenome.net/calypso/) (Zakrzewski et al., 2016).

3. Results

3.1. Sequence data

The total DNA concentration obtained from the bone samples ranged from 0.3 ng/μl to 58 ng/μl (Table 2). For the corresponding soil samples, the total DNA concentration ranged from 0.45 ng/μl to 9.1 ng/μl. The DNA concentrations for both the bone (sample B11) and soil (sample B_Z) blank samples were undetectable (0 ng/μl).

16S rRNA libraries were successfully constructed for all samples; the total library concentration ranged from 1535 pmol/l (sample Z8) to 17 968.4 pmol/l (sample K11). The sample average read length ranged from 178 bp (sample K11) to 255 bp (sample Z11). The average read length obtained for both the K5 and K6 samples was similar to those obtained for the corresponding soil samples (i.e., 198/210 bp and 212/212 bp for pairs K5/Z5 and K6/Z6, respectively). In contrast, the average read lengths obtained for bone samples K7, K8 and K11 were approximately 30–80 bp shorter than for the corresponding soil samples (Table 2).

For the blank samples, the average read length was 338 bp, which was approximately 1.3–1.9 times greater than those obtained for the biological samples.

3.2. Microbiome analysis

During the metagenomics analysis of the sequencing data, nine the most represented bacterial phyla that were detected within the samples were Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes, Chloroflexi, Gemmatimonadetes, Planctomycetes, Nitrospirae and Bacteroidetes. These phyla were the most dominant in both the bone and soil samples, although the proportions differed between sample groups and between individual samples (Fig. 1A). Other bacteria accounted for less than 10% of the sequencing reads. A closer analysis of the mean relative abundance of bacterial phyla in the soil samples showed that Proteobacteria was found to be the most represented phylum (mean relative abundance 32.1%), followed by Actinobacteria (23.6%), Acidobacteria (16.8%), Firmicutes (6.3%), Chloroflexi (5.2%), Gemmatimonadetes (4.7%), Planctomycetes (2.9%), Nitrospirae (2.7%) and Bacteroidetes (1.6%). In the bone samples, the most represented phylum appeared to be Firmicutes, with a mean relative abundance of 43.9%; however, pronounced fluctuations of its fraction between bone samples were observed (Fig. 1A). The next most represented phyla in the bone samples were Proteobacteria, Actinobacteria and Acidobacteria, which accounted for 23.9%, 10.1% and 7.0%, respectively, and resembled the trend of the soil samples, followed by Chloroflexi (3.1%), Nitrospirae (2.9%), Planctomycetes (2.8%), Gemmatimonadetes (2.5%) and Bacteroidetes (0.5%).

Statistically significant differences in the mean relative abundance of bacterial phyla between the bone and soil samples were observed for Actinobacteria, Acidobacteria, Firmicutes and Gemmatimonadetes ($P < 0.05$; Fig. 1B). All but one of these bacterial phyla were more abundant in soil samples; the Firmicutes phylum was more abundant in the bone samples than in the corresponding soil samples ($P = 0.0337$).

By comparing the soil samples and the bone samples, it can be seen that, on the phylum level, the microbiome composition of the soil samples followed a more uniform pattern regardless of the burial place and related characteristics (Fig. 1A). This regularity was further observed through taxa distance heatmap analysis, where the soil samples tended to have a closer relation between their phyla patterns (Fig. 1C). Considering the similarity of the phyla patterns within the bone-corresponding soil pairs, the most similar pair was K8-Z8. Overall, based on the heatmap analysis and the relative microbiome phyla patterns, bone and soil samples K5, K7, K8, Z7, Z8, Z6, Z5 and Z11 clustered together, while two bone samples, K11 and K6, were placed outside this cluster (Fig. 1C).

Table 2
Sequenced sample data.

	Sample Nr									
	K5	K6	K7	K8	K11	Z5	Z6	Z7	Z8	Z11
DNA concentration (ng/μl)	14.1	0.3	0.8	5.37	58.0	7.9	9.1	2.8	1.5	0.45
Library concentration (pmol/l)	17 186.2	9771.6	8262.1	13 602	17 968.4	5630.6	10 830.5	3435	1535	8124.3
Average read length (bp)	198	212	216	214	178	210	212	254	243	255
No of sequenced reads prior to data analysis	161 818	111 099	98 562	227 637	80 006	282 151	314 069	577 032	360 010	242 511
No. of reads mapped to 16SrRNA sequence	161 450	111 070	98 450	227 330	79 235	281 948	313 766	576 417	359 766	242 459

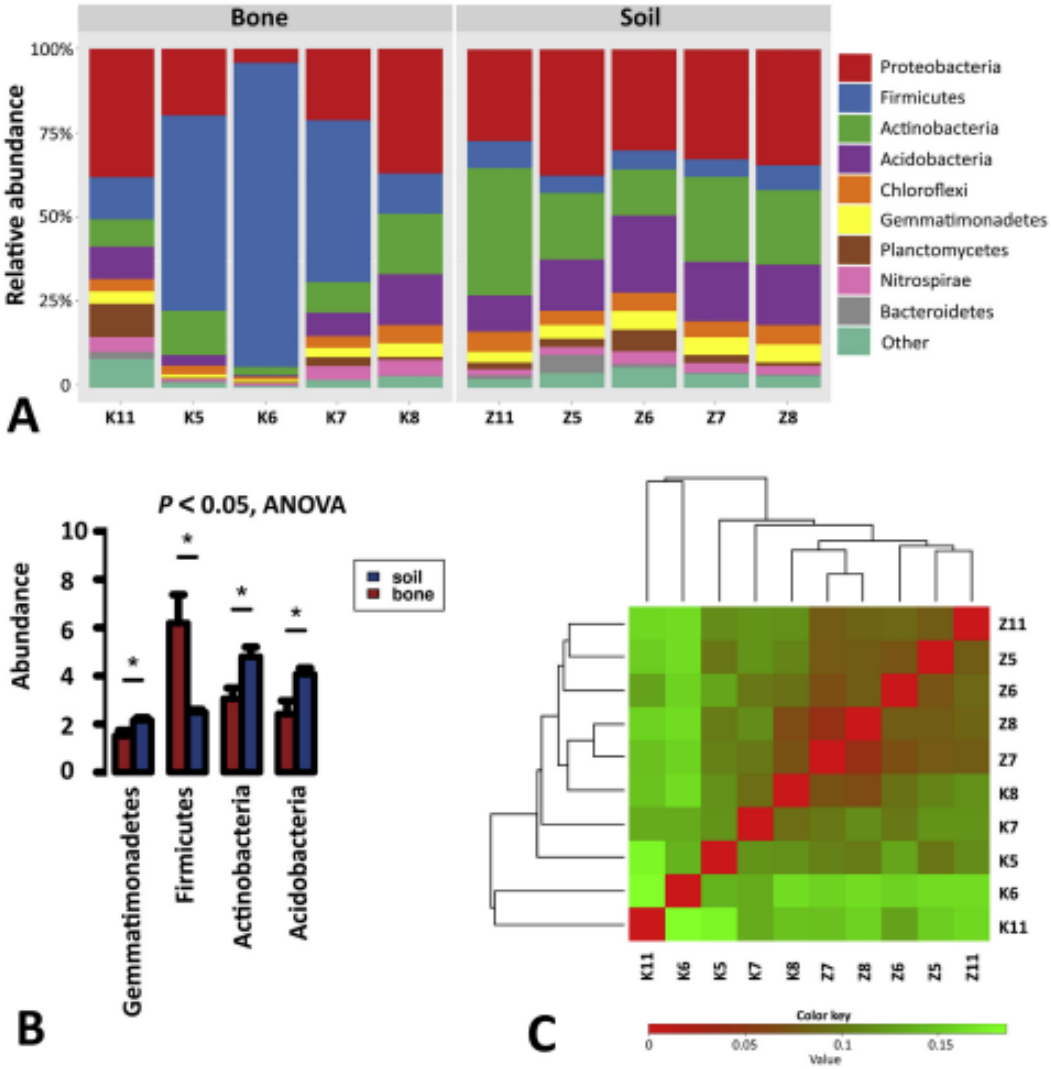

Fig. 1. Phylum-level comparison between bone and soil microbiota. A. Stacked plots of the taxonomic classification at the phylum level using the 16S metagenomes. The relative abundances of the most abundant phyla are shown. B. Comparison of the relative abundances of the bacterial phyla between bone and soil samples. Asterisks denote significant differences ($P < 0.05$) between individual groups. C. Heatmap analysis and clustering dendrogram. The red colour represents the closest resemblance between samples based on the bacterial phyla profiles, while the green colour represents the distance between samples. Samples with more similar microbial populations are clustered closer together. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The microbial alpha diversity (assessed by the Shannon index) on the phylum level did not differ significantly for the bone and soil samples ($P > 0.05$; Fig. 2A, D). Similarly, no significant differences in alpha diversity were observed when the samples were grouped based on the cemetery or the presence/absence of additional burials above the studied ones (Fig. 2B and C). At the genus level, the Shannon index was slightly decreased for the soil samples in comparison to the bone samples and increased in the samples from St. Peter's Church cemetery in comparison with the Dome Square cemetery; however, statistical significance was not reached ($P > 0.05$; Fig. 1D and E).

Further, ANOSIM was carried out using Bray–Curtis similarities to examine the degree of separation between the bacterial communities associated with each group of samples. The ANOSIM indicated significant differences between the bone and soil samples at both the phylum and genus levels ($P < 0.05$; Fig. 3A, D). By contrast, no significant differences in beta diversity were observed when samples were grouped based on the cemetery or the presence/absence of additional above burials ($P > 0.05$; Fig. 3B–F).

Microbial profiles obtained from the blank controls for both the bone and soil samples were clearly distinct from the archaeological samples. The laboratory blank controls were largely dominated by a single phylum, Proteobacteria (Supplementary Fig. 1). This result is similar to observations recently reported for microbial DNA contamination within an ultraclean laboratory with "DNA-free" reagents (Weyrich et al., 2019). This result also indirectly indicates the correct setup of our aDNA facility, as Firmicutes were more dominant in the laboratory controls from modern laboratories (Weyrich et al., 2019).

3.3. *Firmicutes phylum analysis*

As the Firmicutes phylum was significantly more abundant in the bone samples than in the corresponding soil samples, the deeper taxonomic level was further investigated. Among Firmicutes, the most represented bacterial genus in the bone samples appeared to be

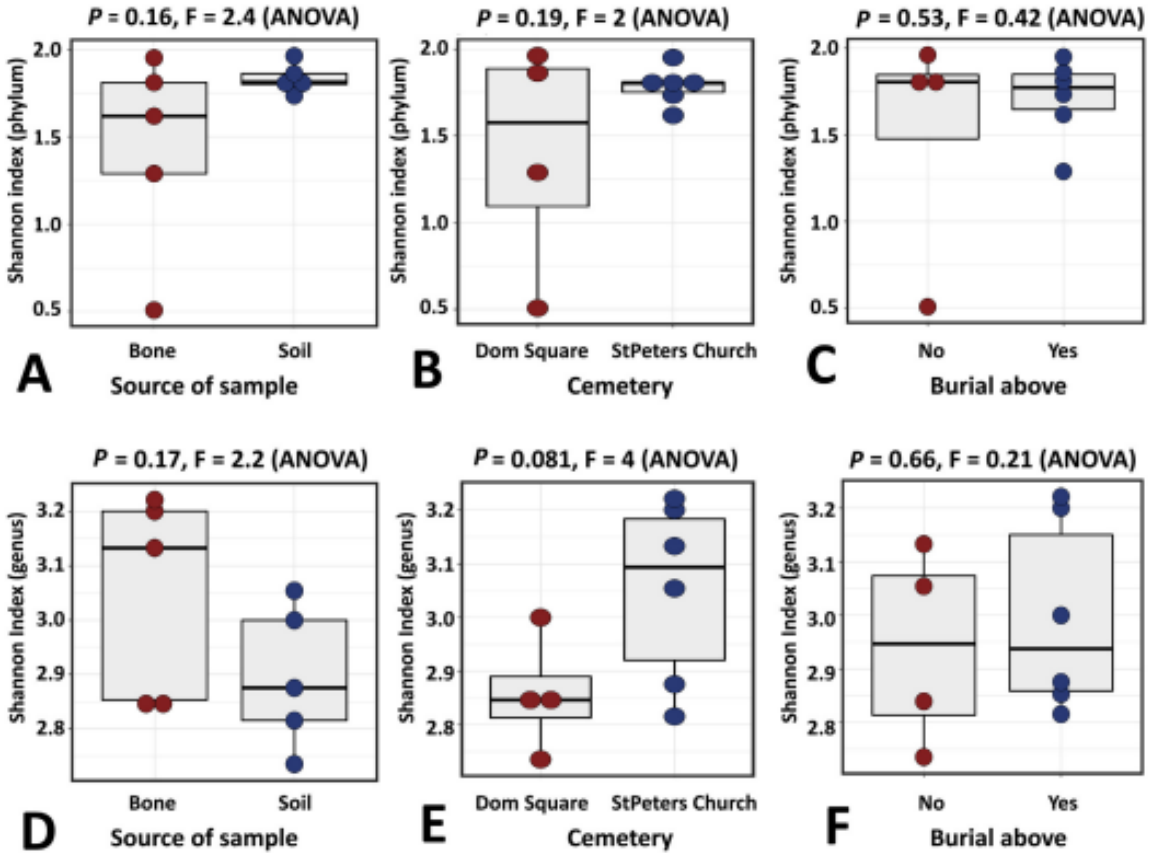

Fig. 2. Microbial Shannon diversity analysis. P values are indicated. A. Phylum level, comparison between soil and bone samples. B. Phylum level, comparison between cemeteries. C. Phylum level, comparison between different burial environments. D. Genus level, comparison between soil and bone samples. E. Genus level, comparison between cemeteries. F. Genus level, comparison between different burial environments.

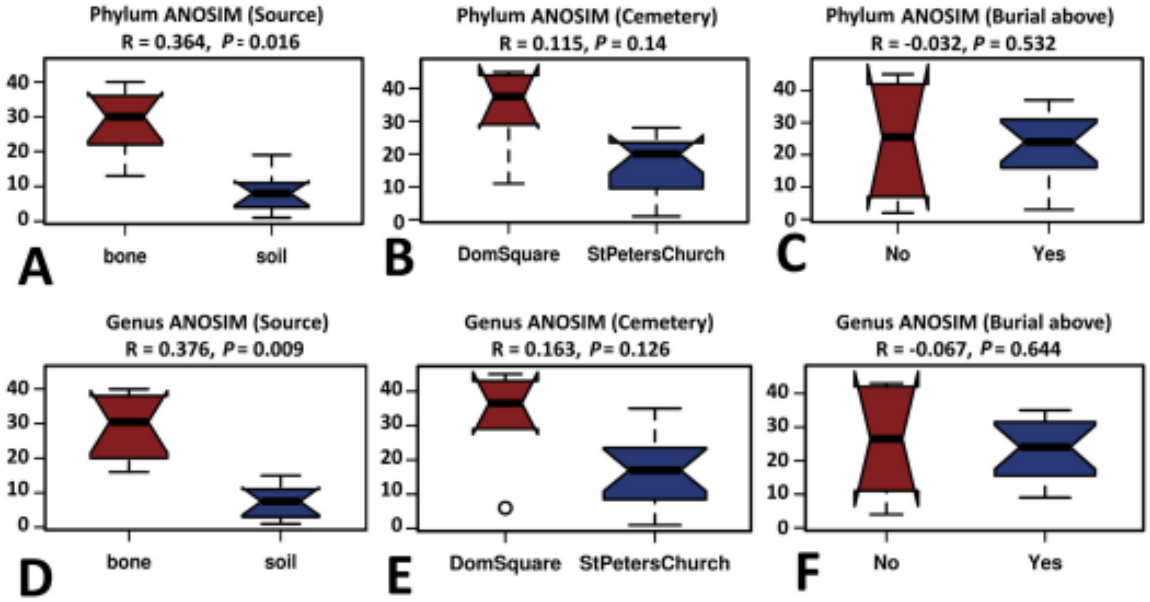

Fig. 3. Microbial beta diversity analysis. *P* values are indicated. A. Phylum level, comparison between soil and bone samples. B. Phylum level, comparison between cemeteries. C. Phylum level, comparison between different burial environments. D. Genus level, comparison between soil and bone samples. E. Genus level, comparison between cemeteries. F. Genus level, comparison between different burial environments.

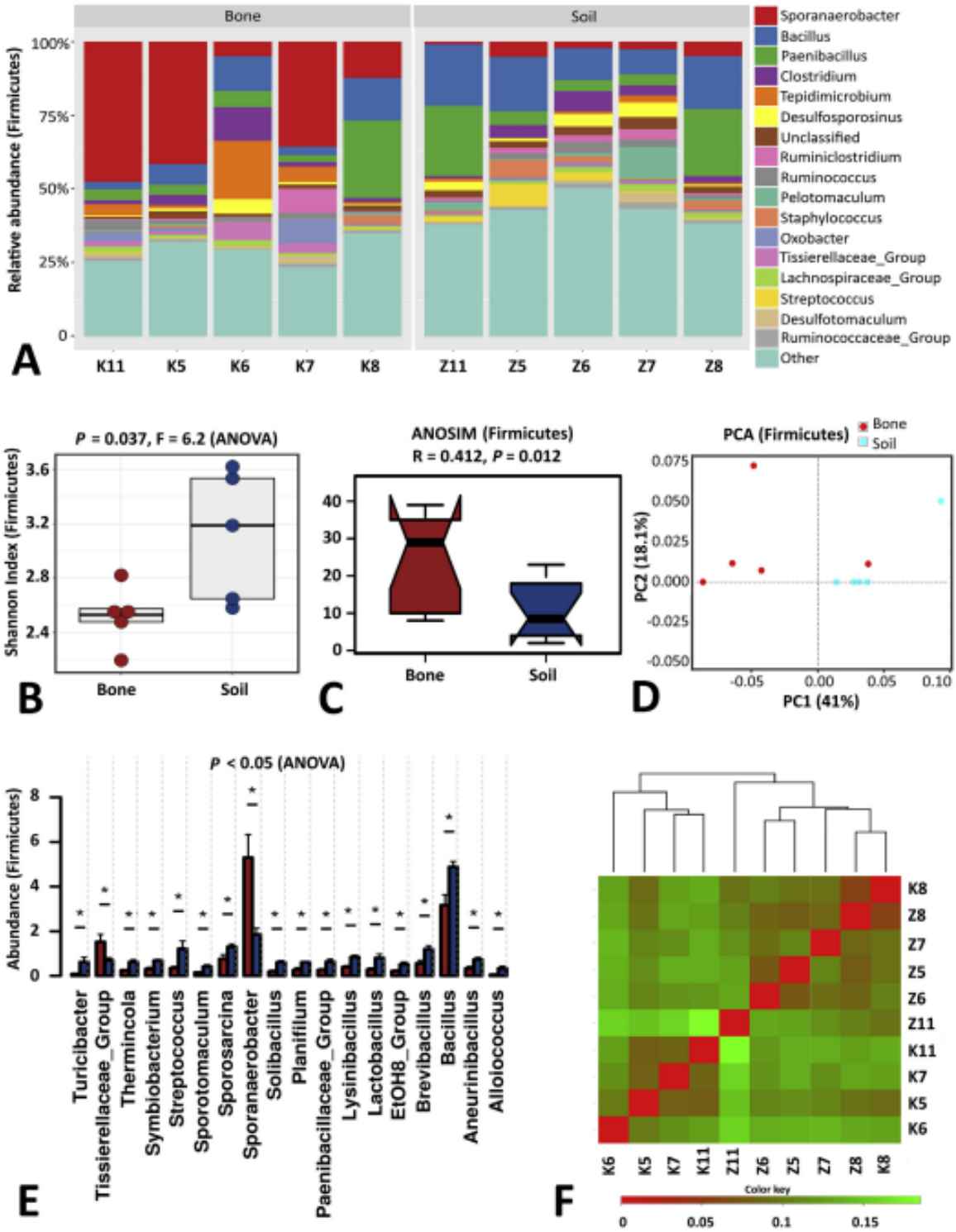

Fig. 4. *Firmicutes* phylum analysis of the bone and soil samples at the genus level. A. Stacked plots of the *Firmicutes* genera using the 16S metagenomes. The relative abundances of the most abundant genera are shown. B. Shannon diversity analysis. C. Beta-diversity analysis. C. Principal component analysis (PCA) of the bone and soil samples. D. Comparison of the relative abundances of the *Firmicutes* genera between bone and soil samples. Asterisks denote significant differences ($P < 0.05$) between individual groups. D. Heatmap analysis and clustering dendrogram. The red colour represents the closest resemblance between samples based on the *Firmicutes* genera profiles, while the green colour represents the distance between samples. Samples with more similar microbial populations are clustered closer together. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Sporanaerobacter, with a mean relative abundance of 28.4%, followed by *Paenibacillus* (8.2%), *Bacillus* (7.8%) and *Tepidimicrobium* (6.1%) (Fig. 4A). The most represented genera in the soil samples were slightly different; the most abundant were *Bacillus* (15.4%) and *Paenibacillus* (11.8%), followed by *Clostridium* (3.6%), *Sporanaerobacter* (3.1%), *Pelotomaculum* (3.1%) and *Desulfosporosinus* (2.8%). The differences in the Firmicutes phylum community between the bone and soil samples were further confirmed by alpha- and beta-diversity analyses, as both the Shannon index and ANOSIM R values were statistically significant ($P < 0.05$) (Fig. 4 B, C).

The beta diversity was further evaluated by clustering using PCA. Based on the calculated organism proportions, two clusters were separated; bone samples K5, K6, K7 and K11 formed one group, and samples Z5, Z6, Z7, Z8, Z11 and K8 formed the other group (Fig. 4D). Similarly, this group separation was observed on the Firmicutes distance heatmap (Fig. 4F). The heatmap also showed a very close correlation between samples K8 and Z8, while all other bone-soil sample pairs appeared to be more distant from each other. The lowest similarity was observed for the K11-Z11 sample pair.

At the genus level, we identified several microbial taxa that were significantly more abundant in the soil samples than in the bone samples and vice versa (Fig. 4E). In the soil samples, a higher abundance of the following genera was observed: *Turicibacter*, *Thermincola*, *Symbiobacterium*, *Streptococcus*, *Sporotomaculum*, *Sporosarcina*, *Solibacillus*, *Planifilum*, *Paenibacillaceae* group, *Lysinibacillus*, *Lactobacillus*, EtOH8 group, *Brevibacillus*, *Bacillus*, *Aneurinibacillus* and *Alloiococcus*. The microbiota of the bone samples showed a remarkably higher abundance of the *Sporanaerobacter* and *Tissierellaceae* group genera ($P < 0.05$).

4. Discussion

The aim of this study was to identify and compare the microbial communities in samples of ancient 15th - 17th century human archaeological bone remains and in the corresponding soil samples from two medieval cemeteries in Riga, Latvia. The main goal was to evaluate the impact of environmental bacterial contamination from the surrounding soil on this type of archaeological material. Overall, the results showed a close correlation with the typical composition of the soil microbiome. However, some exceptions were observed.

Several studies have been conducted in the field of soil microbiome investigation, and some of the most common soil bacteria phyla are believed to be Proteobacteria, Actinobacteria, Acidobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Cytophagales, Gemmatimonadetes, and Firmicutes (Janssen, 2006; Buckley and Schmidt, 2003). However, these phyla are also members of the human microbiome (Hollister et al., 2014). Considering our results of microbial communities in the soil samples, the present phyla resembled the usual soil microbiome abundance in both composition and proportions; Proteobacteria, Actinobacteria, and Acidobacteria were the most abundant bacterial phyla, followed by common soil bacteria phyla such as Firmicutes, Chloroflexi, Gemmatimonadetes and Planctomycetes (Dunbar et al., 1999; Winding et al., 2005).

However, our samples exhibited a relatively high proportion of the Nitrospirae phylum (the mean relative abundance in soil samples was 2.7%; in bone samples, 2.9%), which is not usually mentioned as a common member of soil microbial communities. *Nitrospira* is a nitrite-oxidizing bacterium (NOB) that obtains energy by oxidizing inorganic nitrogen compounds (Lücker et al., 2010). Most commonly, bacteria of the Nitrospirae phylum are found together with other nitrifying bacteria in areas where extensive protein decomposition occurs, and *Nitrospira* is considered to be the most common and abundant NOB in wastewater treatment systems (Daims et al., 2001; Yao and Peng, 2017).

Our observation could be explained by the fact that soil microbiome composition changes with regard to environmental conditions, cultivation technique, and water content (Dunbar et al., 1999; Moreno-Espíndola et al., 2018). On the other hand, in a recent study, Nitrospirae was one of the major phyla with a relatively high abundance throughout the soil profile except for the surface soil (Steger et al., 2019). Thus, this parameter might be a distinctive attribute of the burial environment and/or urban cemeteries, although further investigations are required to confirm this hypothesis. Additionally, there is a possibility that climate change drivers and their interactions may cause changes in the soil microbial community (Castro et al., 2010). However, the possible impact of climate change on the soil microbial community located deeper under the surface, i.e., in the soil at the burial depth, is much less clear.

Overall, in our study, all identified microbial taxa of the bone samples were coincidental to the corresponding soil samples. This result clearly indicates significant infiltration and contamination of archaeological remains with the microbiome of the burial environment. However, the microbiome composition of the soil samples followed a more uniform pattern regardless of the burial place and related characteristics, and differences in microbial community composition between the bone and soil samples were observed at both the phylum and genus levels, as indicated by statistically significant beta-diversity analysis results. Some bacterial taxa were significantly more abundant in soil, while others were more abundant in bone samples. This finding indicates that bone samples act as a niche to preserve microbiome signatures that are different from the burial environment.

The most noticeable shift was observed in the proportion of the Firmicutes phylum, which was significantly more abundant in the bone samples than in the corresponding soil samples ($P = 0.0337$). Moreover, in all bone samples, the relative abundance exceeded the usual Firmicutes percentage of soil microbiomes, which is believed to be less than 10% (Janssen, 2006), reaching a mean value of 43.9%; in our soil samples, the mean abundance was 6.3%.

Firmicutes is a widespread phylum of bacteria found in different environmental conditions; however, it must also be mentioned that this phylum is dominant in the human gut microbiome (Hollister et al., 2014). Firmicutes was also the most abundant microbial group identified in the paleofeces of a pre-Columbian mummy (Santiago-Rodriguez et al., 2016). Bearing in mind the fact that members of the gastrointestinal bacterial community appear in blood within 24 h postmortem while being released into the abdominal cavity (Tito et al., 2008; Bell et al., 1996) together with the fact that Firmicutes is one of the predominant microbial taxa for several decomposing human organs including bones (Javan et al., 2019), it could be suggested that the excess amount of Firmicutes phylum bacteria found in our archaeological samples might be the remains of the individual's gut microbiome.

To test this hypothesis, Firmicutes phylum data were extracted from all sample data and analysed separately to the deeper taxonomic level. By analysing Firmicutes phylum to the genus level, several observations were made. First, the most represented bacterial genera in both the soil and bone samples were common soil genera, and all detected Firmicutes-related genera were coincidental in the bone and soil samples. However, significant diversity in the microbial communities was observed, as indicated by both alpha- and beta-diversity indexes. The PCA and heatmap analysis showed a clear segregation of all but one (K8) bone sample from the corresponding soil samples. These results indicated pronounced differences in Firmicutes abundance at the genus level in archaeological samples and burial environments.

The most abundant bacterial genus of the Firmicutes phylum in the bone samples was *Sporanaerobacter*. This result is intriguing because this microbial genus is known to be constantly observed in later stages of tissue decomposition under anaerobic conditions (Kim et al., 2017; Yang et al., 2012). The *Sporanaerobacter* genus was also present in our soil samples, but with a significantly lower relative abundance (0.28 vs 0.03, $P < 0.05$). The genus *Sporanaerobacter* was identified in the human gut microbiome in pre-Columbian mummies (Santiago-Rodriguez et al., 2016). Considering the fact that the bone samples were exposed to soil for a long period of time, some bacteria may indeed

infiltrate the bone matter through the process of tissue decomposition and may stay trapped inside. The next most abundant genera in bone samples were *Paenibacillus* and *Bacillus*. Both were also detected in soil samples and are known to be typical soil genera. The list of the six most abundant bacterial genera in the bone samples also included the *Tepidimicrobium*, *Clostridium* and *Oxobacter* genera, which could be found in a wide range of environments.

Comparing the bone-soil sample pairs, it can be observed that sample pair K8-Z8 exhibited the greatest similarity between sample pair at both the phylum and genus levels. This observation might indicate excess contamination with environmental bacteria. This assumption, in turn, is supported by the fact that bone sample specimen K8 is a rib (Table 1), which is known to be porous with reduced mineral density (Stout, 1978). It was also noted that bone variety, regardless of weathering level, was a significant factor in the success of mitochondrial DNA amplification (Misner et al., 2009). Thus, the rib sample could have been more exposed to environmental bacterial infiltration and deposition, therefore reducing its potential for being a second deposition material for gut microbiota. On the other hand, samples within the pair K11-Z11 were the most distant in their bacterial microbiome abundance. The archaeological sample K11 was obtained from a skull, while K5, K6, K7 were from vertebra, and K8 was from a rib. The skull, particularly petrous parts of the temporal bone, is well known for its ability to preserve endogenous DNA because it is less prone to weathering than other bones (Pinhasi et al., 2015). Environmental bacterial infiltration was still observed in sample K11; however, K11 bone properties, such as density, might have been the reason for the different distributions of bacterial phyla. Thus, infiltration of ancient human microbiome representatives might undergo the same poorly predictable proportion shift.

Several limitations of this study should be outlined. First, it must be taken into account that the amplicon-based approach used in this study could introduce taxonomic bias. aDNA is known to be highly fragmented and rarely exceeds 200 bp in length; thus, the length of polymorphisms in the V regions of the 16S rRNA gene may lead to differential PCR amplification of microbial taxa (Ziesemer et al., 2015). Additionally, microbiome taxonomic profiles from 16S rRNA sequencing could be shifted towards more recent DNA, such as postmortem overgrowth or environmental contamination, and not reflect the ancient microbiome. While in this study we focused on soil microbiota contamination in skeletal remains, a shotgun metagenomics approach is considered preferable for use in ancient microbiome reconstructions (Ziesemer et al., 2015).

Limitations of this study also included a small sample size and a variation in the sampled skeletal elements, thus a systematic assessment of possible correlations between types of bones and metagenomics composition was not possible. However, despite the limitations of a small study, our results demonstrate the infiltration of environmental bacteria into archaeological material and the possible impact of postmortem processes on the microbiome composition in both skeletal remains and burial environments. These findings urge further studies to understand the extent of microbiota plasticity and the overrepresentation of Firmicutes in ancient bone samples due to taphonomic and environmental factors.

5. Conclusions

The analysis of the microbiome profiles of archaeological bone and the corresponding soil samples revealed significant diversity in microbial compositions. It can be concluded that some bacteria may infiltrate the bone matter through the process of tissue decomposition and remain trapped inside for a longer period of time. Thus, environmental bacteria from the surrounding soil and postmortem changes must be considered important impact factors in ancient human microbiome research.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2019.104989.

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II. The Postmedieval Latvian Oral Microbiome in the Context of Modern Dental Calculus and Modern Dental Plaque Microbial Profiles

Highlights:

Part of the results from this publication was used in these theses.

- Microbial profiles from post-medieval dental calculus were investigated.
- Preservation of human oral microbiome patterns was evaluated in historic dental calculus, modern dental calculus and dental plaque and burial environment samples.
- Majority of microbial DNA from historic dental calculus originated from oral microbiome.

genes

MDPI

Article

The Postmedieval Latvian Oral Microbiome in the Context of Modern Dental Calculus and Modern Dental Plaque Microbial Profiles

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Abstract: Recent advantages in paleomicrobiology have provided an opportunity to investigate the composition of ancient microbial ecologies. Here, using metagenome analysis, we investigated the microbial profiles of historic dental calculus retrieved from archaeological human remains from postmedieval Latvia dated 16–17th century AD and examined the associations of oral taxa and microbial diversity with specific characteristics. We evaluated the preservation of human oral microbiome patterns in historic samples and compared the microbial composition of historic dental calculus, modern human dental plaque, modern human dental calculus samples and burial soil microbiota. Overall, the results showed that the majority of microbial DNA in historic dental calculus originated from the oral microbiome with little impact of the burial environment. Good preservation of ancient DNA in historical dental calculus samples has provided reliable insight into the composition of the oral microbiome of postmedieval Latvian individuals. The relative stability of the classifiable oral microbiome composition was observed. Significant differences between the microbiome profiles of dental calculus and dental plaque samples were identified, suggesting microbial adaptation to a specific human body environment.

Keywords: ancient DNA; dental calculus; dental plaque; oral microbiome; metagenomics

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1. Introduction

The oral cavity serves as a gateway to the human body. It is the place where food preprocessing occurs prior to passing it to the stomach and intestinal tract, has direct contact with incoming air on its way to the trachea and lungs, and contains numerous habitats, including the teeth, gingival sulcus, tongue, cheeks, hard and soft palates, and tonsils, which are colonized by bacteria [1]. At present, the oral microbiome is the second most studied human microbiome subtype after the gut microbiome and is known to hold over 700 species of bacteria belonging mostly to the Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes phyla [2]. Our knowledge about oral microbial communities is wide but not complete: approximately 57% of the known bacterial species are named, 13% of the species have been cultivated but not named, and 30% of the species remain uncultivated [3]. The community of oral bacteria in humans is also known to host a range of pathogenic or potentially pathogenic microorganisms when the healthy microbiome balance is disrupted. Bacteria of the oral cavity are known to cause a range of oral infectious diseases, such as tooth decay (caries), gum and root diseases, alveolar osteitis and tonsillitis [1]. It is

also believed that dental plaque bacterial patterns may be linked with the overall health conditions, lifestyle and dietary preferences of the host [4].

Recent advantages in paleomicrobiology allow us to investigate the evolution of oral microbial ecologies that can contribute to a medical understanding of modern health and nutrition [5]. Ancient dental calculus—calcified oral plaque biofilm—is one of the best archaeological materials to provide us with information related to the ancient human microbiome and its interaction with its host. Dental calculus represents over 600 different bacterial taxa that are common to saliva and dental plaque [1,6,7]. However, searching for human oral microbiome patterns within ancient dental calculus microbial profiles demands a careful interpretation of modern versus ancient microbiome differences. Modern oral microbiome research operates mostly with living bacterial biofilms because dental calculus is not as widespread as it was historically. At the same time, specific differences in bacterial patterns have been discovered between dental plaque and dental calculus, which can be explained by plaque maturation processes [8].

Overall, the application of metagenomic technologies has provided a new tool to directly access the genetic information of a microbial community in historical samples, allowing us to address a number of questions, such as the exact nature of the bacteria present in particular historical periods and geographical regions.

Here, using metagenome analysis, we aimed to investigate the microbial profiles of historic dental calculus retrieved from archaeological human remains from postmedieval Latvia dated 16–17th century AD. We evaluated the preservation of human oral microbiome patterns in historic samples by comparison to modern human dental plaque and calculus samples and burial soil microbiota and concluded that the majority of microbial DNA in historic calculus originates from the oral microbiome with little impact of the burial environment. We also tested the associations of oral taxa and microbial diversity in historic dental calculus with specific characteristics.

2. Materials and Methods

2.1. Archaeological Sample Characteristics, Collection and Burial Site Data

During this study, 15 historic human dental calculus samples were examined, representing 6 Latvian cemeteries in 4 cities: the capital Riga (Dom Square cemetery, 56.94902, 24.10473; St. Peter's Church cemetery, 56.94752, 24.10928; St. Gertrude's Church cemetery, 56.95799, 24.12172), Cesis (St. John's Church cemetery, 57.31213, 25.27168), Kuldiga (Church of the Holy Trinity, Roman Catholic Church, 56.96765, 21.96943) and Jelgava (St. Trinity's Church, 56.65239, 23.72897). All samples were dated to 16–17th century AD (Table 1).

Table 1. Characteristics of archaeological samples.

Sample ID	City	Cemetery	Century, AD	Year of Excavation	Age (Years)	Sex	Lifestyle	Possible Diseases	Tooth Type
ZA_1C	Riga	Dom Square	16–17th	1986	30–40	Male	Town/city, middle classes	Inflammation of the clavicle	Molar
ZA_3C	Riga	Dom Square	16–17th	1986	45–50	Female	Town/city, middle classes	Caries	Incisor
ZA_4C	Riga	St. Peter's Church	16–17th	2004	30–35	Male	Town/city	-	Incisor
ZA_5C	Riga	St. Peter's Church	16–17th	2004	25–30	Male	Town/city	Fractured rib and left arm's fracture	Incisor
ZA_6C	Riga	St. Peter's Church	16–17th	2004	20–25	Male	Town/city	Non-specific inflammation of the lower leg bone surface	Premolar
ZA_7C	Riga	St. Gertrude's Church	16–17th	2006	14–15	Unknown	Countryside, commuter town	-	Incisor

Table 1. *Cont.*

Sample ID	City	Cemetery	Century, AD	Year of Excavation	Age (Years)	Sex	Lifestyle	Possible Diseases	Tooth Type
ZA_8C	Riga	St. Gertrude's Church	17th	2006	45–50	Male	Countryside, commuter town	Deforming arthrosis (joints)	Incisor
ZA_9C	Riga	St. Gertrude's Church	16–17th	2006	25–30	Male	Countryside, commuter town	Vertebral fracture with local inflammation	Molar
ZA_10C	Riga	St. Gertrude's Church	16–17th	2006	55–60	Male	Countryside, commuter town	Caries, tooth root abscess, arthritis in joints	Molar
ZA_11C	Cesis	St. John's Church	17th	2015	35–40	Male	Town/city, aristocracy	Multiple tooth decay, toe osteomyelitis	Incisor
ZA_12C	Cesis	St. John's Church	17th	2015	35–40	Female	Town/city, aristocracy	Non-specific inflammatory process on the surface of the leg bones	Premolar
ZA_20C	Kuldiga	Church of the Holy Trinity, Roman Catholic Church	16–17th	2015	45–50	Female	Town/city, lower classes	-	Molar
ZA_22C	Kuldiga	Church of the Holy Trinity, Roman Catholic Church	16–17th	2015	30–35	Female	Town/city, lower classes	-	Incisor
ZA_29C	Jelgava	St. Trinity's Church	16–17th	2009	35–40	Female	Town/city, aristocracy	Caries, arthritis in joints, non-specific inflammation	Premolar
ZA_C22	Riga	St. Gertrude's Church	17th	2006	40–50	Unknown	Countryside, commuter town	Deforming arthrosis, pelvic joint	Incisor

Most individuals were in their young adulthood to middle age (20–60 years of age) at the time of death. Sample ZA_7C was suspected to represent a teenager (14–15 years of age). Prior to dental calculus sample collection, archaeological skeletons were inspected for the presence of any disease-specific leisure signs. Mouth cavities were inspected for the presence of oral disease lesions [9]. Four archaeological skeletons were found to exhibit tooth decay signs, 10 skeletons exhibited specific and nonspecific bone lesions, and for four skeletons, no signs of diseases were observable (Table 1). The teeth and the alveolar bone appeared to be macroscopically sound without traces of periodontal disease. To determine the burial period of the samples, the stratigraphy method was used together with the evaluation of archaeological finds [10,11]. The approximate age at death was determined by evaluating degenerative changes in the pubic symphysis and using the auricular surface standard method [12,13].

Random soil samples were collected together with bone samples during the excavation process to evaluate the microbiome composition of the burial environment. Burial soil samples were collected from two cemeteries in Riga, Latvia: Dome Square cemetery (samples Z5 and Z6_sk) and St. Peter's Church cemetery (samples Z7 and Z7_sk). Soil samples were collected at the burial depth from the middle section of the skeleton, i.e., between the ribcage and the pelvis, approximately 5–10 cm above the bones. All samples

were packed in plastic bags and stored separately from the bone samples under the same conditions to avoid any further contamination.

2.2. Modern Dental Calculus and Modern Dental Plaque Sample Collection

Modern dental supragingival calculus samples (n = 4) were collected at the Institute of Stomatology (Riga Stradins University, Riga, Latvia) by a professional dentist during the process of routine dental cleaning (Table 2). Dental calculus samples were collected with the use of a dental scaler. The exact location of the sample taken was selected arbitrarily by the dentist. Modern dental supragingival plaque samples (n = 20) were collected by rubbing a cotton swab over the supragingival sites of the teeth. All samples were collected with patient consent, and the study was reviewed and approved by Riga Stradins University Research Ethics Committee, decision no. 6-3/4/5 (25 April 2019).

Table 2. Characteristics of modern samples.

	Sample Type	Sex	Teeth Health *	Age (Years) **
MZ2	Supragingival dental plaque	Male	healthy	23
MZ3	Supragingival dental plaque	Male	treated	23
MZ4	Supragingival dental plaque	Male	treated	28
MZ5	Supragingival dental plaque	Female	treated	23
MZ6	Supragingival dental plaque	Male	treated	24
MZ7	Supragingival dental plaque	Female	treated	24
MZ8	Supragingival dental plaque	Male	treated	24
MZ9	Supragingival dental plaque	Male	treated	22
MZ10	Supragingival dental plaque	Female	healthy	23
MZ11	Supragingival dental plaque	Female	treated	22
MZ12	Supragingival dental plaque	Female	treated	22
MZ13	Supragingival dental plaque	Female	healthy	22
MZ14	Supragingival dental plaque	Female	treated	23
MZ15	Supragingival dental plaque	Female	treated	23
MZ16	Supragingival dental plaque	Female	treated	22
MZ17	Supragingival dental plaque	Female	treated	22
MZ18	Supragingival dental plaque	Female	treated	19
MZ19	Supragingival dental plaque	Male	treated	22
MZ21	Supragingival dental plaque	Female	healthy	23
MZ22	Supragingival dental plaque	Female	healthy	20
S1	Supragingival dental calculus	Male	treated	N/A
S2	Supragingival dental calculus	Male	treated	N/A
S4	Supragingival dental calculus	Female	treated	N/A
S5	Supragingival dental calculus	Male	treated	N/A

* treated: dental fillings were present. ** N/A—not available.

2.3. DNA Isolation

Ancient DNA (aDNA) handling in laboratory conditions requires special care and precautions to eliminate all possible contamination from modern DNA sources. This study strictly followed specific guidelines developed exclusively for aDNA research [14,15]. aDNA handling protocols consist of actions prescribing facility preparation prior to aDNA handling, instrument treatment, facility worker equipment and archaeological material processing. Ancient DNA facility preparation required regular ultraviolet (UV) light irradiation and weekly surface and floor cleaning actions with 5% sodium hypochloride (NaClO) solution. All instruments involved in aDNA procedures were treated similarly by washing with 5% NaOCl solution and irradiating with UV light before and after each procedure. Archaeological samples were processed one at a time.

The aDNA facility consisted of three strictly separated chambers, each serving its defined purpose. Archaeological material preprocessing and pulverized sample incubation occurred in one facility chamber, and another two chambers were devoted to aDNA isolation and library preparation. The fourth chamber (buffer zone) separated the aDNA facility from the rest of the laboratory.

Archaeological tooth samples carrying the desired dental calculus remains were first immersed in 5% NaOCl solution, rinsed with nuclease-free water and irradiated for 2 h with UV light (6 J/cm2 at 254 nm). Samples were then left to dry overnight at room temperature. The next day, dental calculus was cautiously removed from the surface of the teeth with a scalpel and was ground inside a tube with a sterile microbiological stick. Laboratory blank samples (BC and BC_sk) were processed simultaneously with archaeological samples. DNA extraction was performed as described previously [16,17].

Burial soil and modern dental calculus samples were processed in another facility to avoid possible cross-contamination with aDNA samples. Burial soil samples that were chosen to represent the soil microbiome of the burial environment of St. Peter's Church cemetery (samples Z5 and Z6_sk) and St. Gertrude's Church cemetery (samples Z7 and Z7_sk) underwent the same procedures of DNA extraction and purification as the aDNA samples. Modern dental calculus samples were washed with 5% NaOCl solution and were then rinsed with nuclease-free water. Furthermore, together with the modern dental plaque samples, modern dental calculus samples underwent a DNA extraction process.

All resultant DNA samples were inspected with a Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA) to estimate the resultant DNA concentration (Table S1, Supplementary Materials).

2.4. Library Preparation and Shotgun Metagenomics Sequencing

Before library preparation, modern dental calculus, modern dental plaque DNA samples, and burial soil DNA samples Z6_sk (St. Peter's Church cemetery) and Z7_sk (St. Gertrude's Church cemetery), underwent an additional DNA fragmentation step using the Ion Shear™ Plus Reagent Kit (Ion Torrent™, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The fragmentation conditions were selected according to the desired fragment size (150–250 bp).

For the historic dental calculus samples and for burial soil samples Z5 (St. Peter's Church cemetery) and Z7 (St. Gertrude's Church cemetery), the DNA fragmentation step was omitted to ensure the capture of short DNA fragments that are believed to represent aDNA [18–20]. DNA samples underwent a size-selection procedure to remove DNA fragments larger than 250 bp using NucleoMag®NGS Clean-up and Size Select magnetic beads (Macherey-Nagel), and for library preparation, an Ion Plus Fragment Library Kit (Ion Torrent™) was used. To evaluate sample contamination from laboratory sources, two laboratory control samples were processed with the historical DNA samples and sequenced: aDNA extraction blank sample (BC) and aDNA extraction blank sample, which also underwent a DNA fragmentation step (BC_sk).

Preparation of all sequencing libraries followed the same steps regardless of sample origin. Specific barcodes were ligated, and libraries underwent amplification and quality

assessment using an Agilent High Sensitivity DNA Kit and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. Sequencing was performed on an Ion ProtonTM System (Ion Torrent™, Thermo Fisher Scientific, Waltham, MA, USA).

All raw historic calculus DNA sequencing data are publicly available at the ENA under accession PRJEB40382.

2.5. Sequencing Data Analysis

Sequencing data preprocessing on the local Ion Torrent Proton server included initial quality control steps as well as data assignment to each individual sample. Barcodes and sequencing adapters, together with polyclonal and low-quality sequences, were filtered by Proton software during the first post-sequencing data handling step. The resultant data were exported for further manipulations in the form of BAM files. The resulting exported BAM files were initially quality-processed using the Galaxy public server [21,22]. Briefly, overrepresented sequences were removed, and reads with quality PHRED scores <20 were excluded. Sequencing data taxonomic assignment was performed with Kraken2 v2.0.7 using the standard Kraken2 database [23]. Bracken (Bayesian Reestimation of Abundance with Kraken) was used to compute the abundance of species in DNA sequences from a metagenomics sample [24].

Pavian R application [25] was used to further manipulate Kraken/Bracken taxonomy report files, generate quality assignment and prepare data for statistical analysis and representation, which was done using the MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/ (accessed on 23 December 2020)) web application [26,27].

The Bayesian analysis-based program SourceTracker [28] was used to evaluate the possible source of predominant microbial signatures of historic dental calculus samples to enable oral microbiome preservation assessment and to track the influence of exogenous microbial contamination. All source files, which were included in the pipeline, were defined from these study samples. There were five sources defined: modern supragingival calculus, modern supragingival plaque, two aDNA extraction blanks and burial soil. The open-source R package decontam (https://github.com/benjjneb/decontam (accessed on 23 December 2020)) [29] was used for the identification and removal of laboratory contaminants in metagenomics data. Low-abundance species were removed by applying a hard cutoff (0.001% abundance).

The authenticity of historic specimen microbiome data was confirmed using DNA damage patterns. This method is based on the hypothesis that DNA deamination rates increase over time [19], and characteristic features of damaged DNA patterns may confirm the origin of the DNA. For this purpose, read files of historic dental calculus samples were analyzed using MALT 0.5.0 [30] (https://software-ab.informatik.uni-tuebingen.de/download/malt/welcome.html (accessed on 23 December 2020)), using all complete bacterial genomes available from NCBI Assembly in August 2020 as a reference. Bacterial reads were extracted using SAMtools, mapped to the reference genome of the prevalent oral microorganism *Olsenella* sp. oral taxon 807 and *Actinomyces* sp. oral taxon 414, and DNA deamination rates were calculated using mapDamage [31] by the EAGER pipeline [32].

2.6. Statistical Analysis

Intergroup differences in alpha diversity were assessed by the Shannon diversity index. Beta diversity was tested by permutational multivariate analysis of variance (PERMANOVA), a nonparametric multivariate statistical test [33] presented by principal coordinates analysis (PCoA). Hierarchical clustering was visualized by dendrogram and heatmap analysis using the Bray–Curtis similarity index and Ward clustering algorithm.

Intergroup differences at the species level were analyzed using the Kruskal–Wallis test and the linear discriminant analysis (LDA) effect size (LEfSe) method [34] with default settings on the MicrobiomeAnalyst website; the threshold on the logarithmic LDA score

for discriminative features was set to 2.0, and the false discovery rate (FDR)-adjusted p value cutoff was set to 0.05.

3. Results

3.1. Sequencing Data and Ancient DNA Authentication

In total, a set of 43 DNA samples was analyzed: historic supragingival calculus (15 samples), modern supragingival calculus (4 samples), modern supragingival plaque (20 samples) and burial soil (4 samples). A total of 0.11 billion sequences were generated, with an average of 2.6 million reads (standard deviation (SD) = 1.3 million reads) per sample (Table S1, Supplementary Materials). Among them, 19.34% of the reads were classified to the bacterial species level, with an average of 0.51 million reads (65248–1928089; SD 0.40 million reads) per sample.

For both blank samples, despite the sufficient sequencing depth, much lower amounts of classified reads were obtained: only 2260 (1.27%) and 688 (1.29%) reads were classified to the bacterial species level for the aDNA extraction blank samples BC and BC_sk, respectively. A large proportion of the obtained reads in blank samples seem to be sequencing artifacts that could not be classified. In total, in the aDNA extraction blanks, 14 bacterial species were detected; the vast majority of the reads (87.35%) belonged to the *Delftia* genus (Figure S2A, Supplementary Materials). In other studies, low bacterial diversity was routinely obtained from laboratory extraction controls [35], while the *Delftia* genus has been detected in sequenced negative 'blank' controls and detected as a contaminant of amplification kits [36].

SourceTracker analysis demonstrated that the historical dental calculus sample group in this study had a predominant dental calculus microbial signature, indicating sufficient preservation of the oral microbiome in a mineralized oral plaque biofilm (Figure S1, Supplementary Materials). A modern dental plaque signature was also present, but in lower amounts. Traces of exogenous contamination defined from the burial soil source and laboratory blanks were low, indicating a low impact of laboratory and environmental contamination on the study samples.

For the authentication of oral microbiome preservation in historic dental calculus samples, the DNA damage pattern of two of the most prevalent oral microorganisms, *Olsenella* sp. oral taxon 807 and *Actinomyces* sp. oral taxon 414 was evaluated. The analysis revealed signs of cytosine to thymine substitutions at the ends of DNA fragments, characteristic of aDNA (Figures S3 and S4, Supplementary Materials).

3.2. Taxonomical Analysis of Microbial Profiles at the Species Level

Based on decontam analysis and low prevalence (prevalence filter: 0.001%), a total of 1842 OTUs were removed, and 2223 features remained after data filtering within all 43 samples. During the metagenomics analysis of the sequencing data, differences in the overall microbial patterns at the species level were observed for the four sample groups (Figure 1A).

The 10 most abundant bacterial species within the group of modern dental plaque samples were *Veillonella parvula* (14.92%), *Haemophilus parainfluenzae* (12.31%), *Streptococcus mitis* (7.10%), *Streptococcus oralis* (4.37%), *Neisseria mucosa* (4.23%), *Prevotella melaninogenica* (3.46%), *Streptococcus pneumoniae* (2.82%), *Streptococcus sanguinis* (2.56%), *Fusobacterium nucleatum* (2.53%), and *Rothia dentocariosa* (1.97%) (Table S2, Supplementary Materials). In contrast, the 10 most abundant bacterial species within the modern calculus samples were *Propionibacterium acidifaciens* (13.33%), *Parascardovia denticolens* (10.25%), *Scardovia inopinata* (8.57%), *Lautropia mirabilis* (3.66%), *Actinomyces* sp. oral taxon 414 (3.32%), *Pseudopropionibacterium propionicum* (3.06%), *Acinetobacter johnsonii* (2.98%), *Neisseria elongata* (2.94%), *Olsenella* sp. oral taxon 807 (2.03%), and *Streptococcus sanguinis* (2.01%).

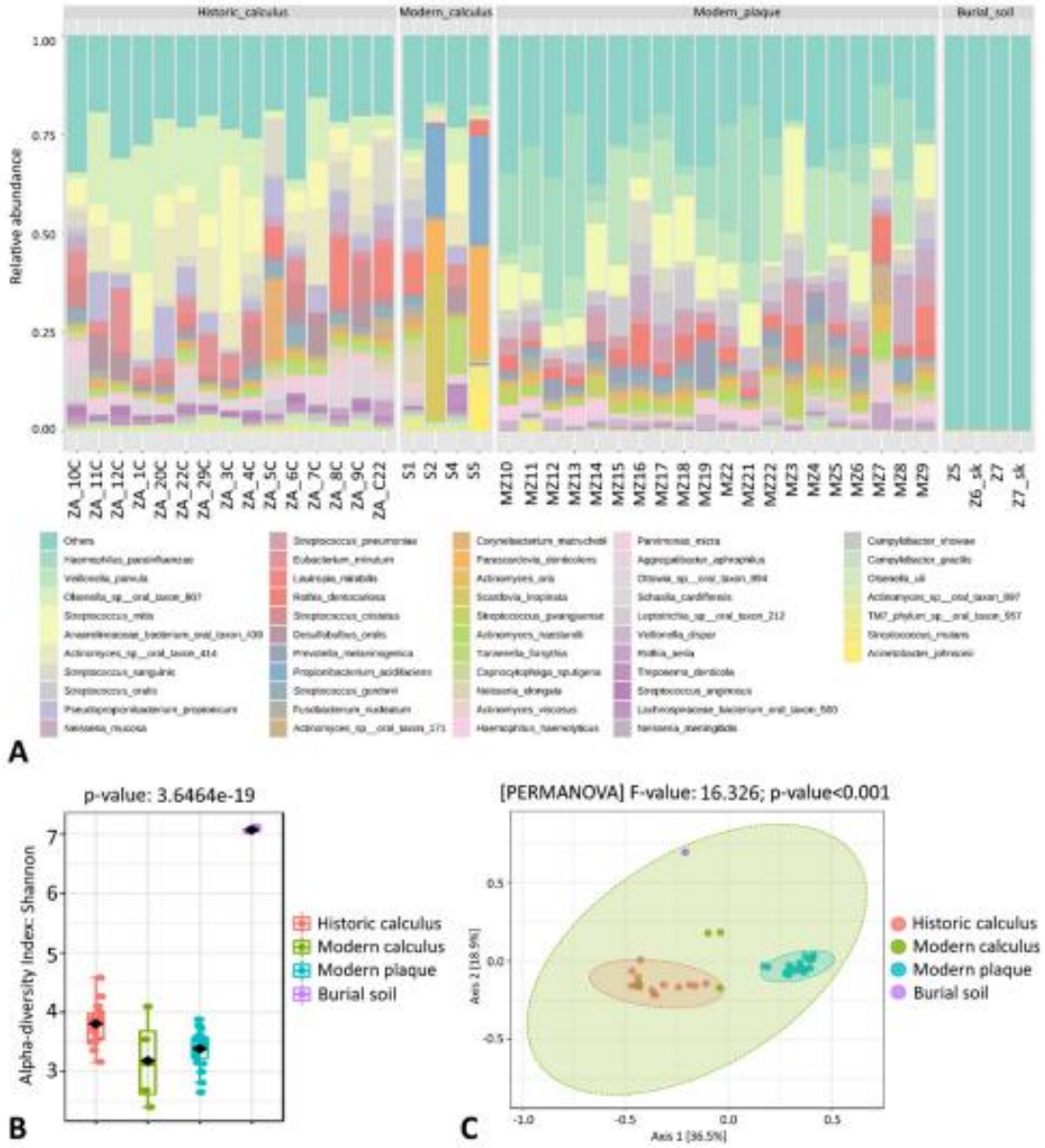

Figure 1. Species-level comparison of bacterial profiles in 16–17th century human archaeological dental calculus, modern dental calculus, modern dental plaque and burial soil samples: (**A**) Stacked plots of the taxonomic classification. The abundances of the most abundant species are shown; (**B**) Shannon diversity analysis; (**C**) Principal coordinates analysis (PCoA) derived from Bray–Curtis distances among samples of the four groups ($p < 0.001$ by PERMANOVA). For each axis, in square brackets, the percent of variation explained was reported.

Historic dental calculus samples represented a slightly different pattern of the most abundant species, the first 10 of which were *Olsenella* sp. oral taxon 807 (11.83%), *Actinomyces* sp. oral taxon 414 (8.29%), *Anaerolineaceae* bacterium oral taxon 439 (7.84%), *Pseudopropionibacterium propionicum* (5.20%), *Streptococcus sanguinis* (4.96%), *Eubacterium minutum* (3.53%), *Desulfobulbus oralis* (3.51%), *Lautropia mirabilis* (3,42%), *Streptococcus cristatus* (2.85%), and *Ottowia* sp. oral taxon 894 (1.82%) (Table S2, Supplementary Materials).

Additionally, the presence of several bacteria that were among the most abundant species in the archaeological calculus samples was detected in the blank samples but in much lower proportions (Figure S2A, Supplementary Materials). As the blank samples were processed simultaneously with the historical dental calculus samples, these results

indicate that the contamination was most likely passed from the archaeological samples to the blank samples.

Burial soil samples showed much higher microbial alpha diversity than the oral microbiome samples (ANOVA, F value = 111.31, $p < 0.001$) (Figure 1B); the ten most abundant species in soil samples were *Sorangium cellulosum* (1.11%), *Polaromonas* sp. JS666 (0.83%), *Luteitalea pratensis* (0.69%), *Gemmatirosa kalamazoonesis* (0.52%), *Rhodopseudomonas palustris* (0.48%), *Streptomyces venezuelae* (0.40%), *Planctomycetes* bacterium ETA A1 (0.39%), *Streptomyces lydicus* (0.39%), *Achromobacter xylosoxidans* (0.39%), and *Streptomyces hygroscopicus* (0.35%), while other species composed 88.28% of the sequencing reads (Figure S2B, Supplementary Materials). The most abundant soil-related bacterial species were also detected in historical dental calculus samples but in much smaller amounts (Table S3, Supplementary Materials).

Microbial beta diversity analysis found significant separation between burial soil, historic dental calculus and modern dental plaque samples (PERMANOVA, F value = 16.326, $p < 0.001$) (Figure 1C). Modern calculus samples were located closer to the historic calculus sample cluster, and dental plaque samples and burial soil samples formed tightly separated aggregates. A dendrogram analysis using the Bray–Curtis Index and the Ward clustering method also showed clear separation of the dental plaque, burial soil and dental calculus samples regardless of the origin, based on the normalized relative abundance of identified bacterial species (Figure 2).

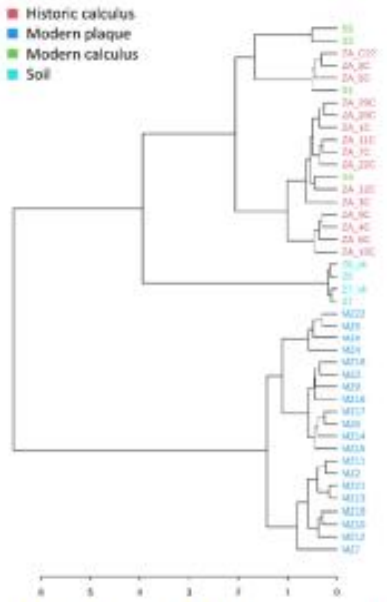

Figure 2. Species-level clustering dendrogram based on the Bray–Curtis index and Ward clustering algorithm. Samples with more similar species profiles were clustered closer together.

LEfSe analysis identified 34 differentially abundant bacterial taxa in the microbiotas of historic dental calculus, modern calculus, modern dental plaque and burial soil samples (LDA score [log 10] > 4.5) (Figure 3). Within the most significant LEfSe results (LDA score [log10] > 5.0) at the species level, we found six microbial species that were mainly attributed to the modern dental plaque sample group (*Haemophilus parainfluenzae*, *Veillonella parvula*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus pneumoniae*, *Prevotella melaninogenica*) and five species were attributed to the historic dental calculus sample group (*Olsenella* sp. oral taxon 807, *Anaerolineaceae* bacterium oral taxon 439, *Actinomyces* sp. oral taxon 414, *Eubacterium minutum* and *Desulfobulbus oralis*) (Figure 3).

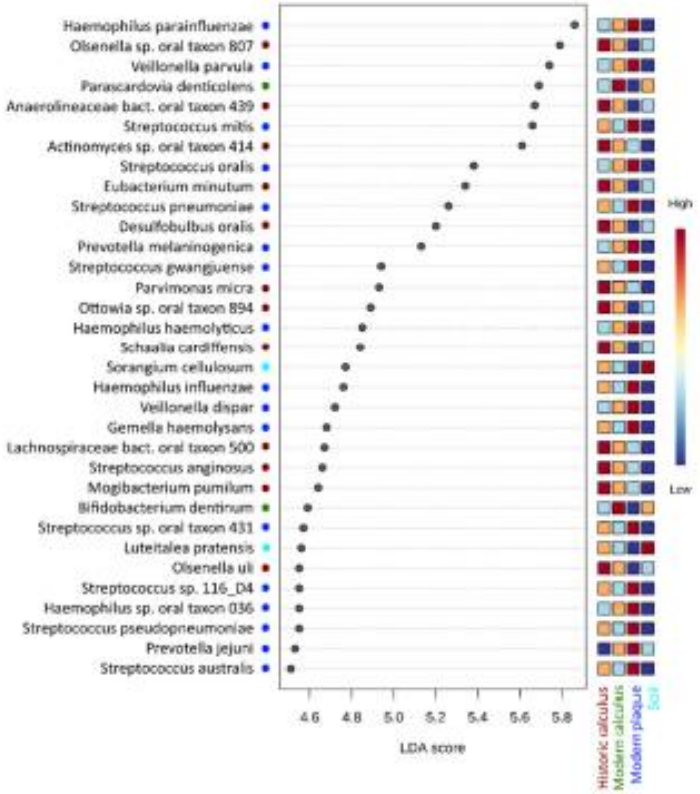

Figure 3. Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) identified species that enabled discrimination between the microbiotas of ancient calculus, modern calculus, modern plaque and burial soil samples. False Discovery Rate (FDR)-adjusted p value cutoff: 0.05; logarithmic LDA score $\geq$ 4.5.

3.3. *Taxonomic Analysis of Microbial Profiles of Historic Dental Calculus Samples at the Species Level*

Furthermore, a detailed metagenomic analysis of the microbiome signature of the historical dental calculus samples was performed. As shown in Figure 1A, the number of sequences aligned to particular oral bacteria differed among the subjects analyzed. Sample ZA_5C had a very high level of *Corynebacterium matruchotii* (20.31%), ZA_1C—*Olsenella* sp. oral taxon 807 (33.73%), and ZA_3C—*Anaerolineaceae* bacterium oral taxon 439 (40.12%).

The taxonomic composition of the historic dental calculus samples was studied at the species level based on the sample characteristics, including sex, age group ($\leq$40 years old and >40 years old), possible lifestyle (town and countryside), tooth type and presence/absence of caries. No significant differences in alpha and beta diversity were observed (Figures S5 and S6, Supplementary Materials), and no significant features were detected during LEfSe analysis either.

No oral pathogen species were significantly associated with the prevalence of caries by univariate analysis (Kruskal–Wallis test, false discovery rate corrected p value greater than 0.05), including *Streptococcus mutans* and members of the periodontitis-associated 'red-complex' (*Treponema denticola*, *Tannerella forsythia*, *Porphyromonas gingivalis*) [37].

4. Discussion

In our study, historic dental calculus, modern dental calculus and oral plaque samples were analyzed by a shotgun metagenomics approach. For the first time, oral microbiome patterns of postmedieval Latvian individuals were studied, and the results of this study showed that despite some burial environmental contamination, historic dental calculus samples provide a reliable snapshot of bacterial oral communities from past individuals.

The path of historical bacterial research has various challenges. The implementation of multiple methodologies (from laboratory practices to data analysis quality control pipelines) helps to improve validation of aDNA data in ancient dental calculus samples; however, environmental contamination (from both burial soil and laboratory environments) in samples is a reality. Extraction blank controls should always be included and sequenced together with archaeological samples to monitor in-lab contamination, and caution must be taken in interpreting the data [18]. Moreover, as we observed in our study for the blank controls, low-level contamination can also happen from one sample to another during the workflow, thus especial care should be taken when samples are processed in batches. In our study, we avoided this probable issue by processing the archaeological samples one at a time; however, this approach is time-consuming and could not be feasible on a larger scale.

The analysis of burial soil samples together with archaeological samples was suggested to assess the environmental contamination [38]. In our study, a clear separation of burial soil microbiomes from both ancient and modern oral microbiomes was observed. Several soil bacterial species were detected in ancient dental calculus microbiomes but were nearly absent in modern oral samples, although the ancient and modern calculus samples were clustered tightly together. This finding can be easily explained by the direct impact of the burial environment. However, industrialization, urbanization, and modern food processing have dramatically reduced human contact with soil microorganisms. Traces of dirt may be incorporated into dental calculus over a lifetime of eating food that is not fully cleaned. This problem is further complicated by the fact that some microorganisms tend to colonize multiple environmental niches, including soil and oral cavity. For example, an environmentally ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* is known to be involved in multiple oral infections [39,40]. Additionally, a recent study proposed a novel environmental microbiome hypothesis, stressing a close linkage between the human intestinal microbiome and the soil microbiome, which has evolved during evolution [41]. This theory might also affect the ancient human oral microbiome. However, in our study, we were not able to demonstrate the ancient origin of the soil taxa found in historic calculus samples due to the relatively low sequencing coverage. Additional studies are required that would shed light on this question.

The vast majority of bacterial species detected in historical dental calculus belonged to the oral microflora, and despite some individual variations, the microbiome composition did not differ significantly from that of the modern samples. The similarities between postmedieval and modern calculus samples can be explained by the relatively short time span and the same geolocation. Additionally, the majority of archaeological samples in our study were from individuals who possibly had better access to foods that were linked with higher social status, such as soft dietary carbohydrates (finely ground bread, sweets) and meat [42]. On the other hand, we did not identify significant differences between ancient samples that were grouped based on sex, age, and lifestyle. This result is consistent with previous studies [8,38,43] suggesting the relative stability in the composition of the oral microbiome at definite time points.

The most abundant bacterial taxa detected in our historical calculus samples included several commensal bacterial species that are commonly found in the human oral cavity, such as *Streptococcus sanguinis*, *Streptococcus cristatus* and *Lautropia mirabilis*. The periodontal pathobiont *Desulfobulbus oralis* was present among the 10 most abundant bacterial species from postmedieval dental calculus samples, which is known for its ability to trigger a proinflammatory response in the oral epithelium [44]. Additionally, many oral pathogens that are involved in the etiology of caries, such as *Streptococcus mutans*, or periodontal disease, including three Gram-negative species known as the 'red-complex'—*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*—were detected in our historical calculus samples. While archaeological samples included in our study were without clear evidence of periodontal disease, the presence of periodontal organisms is not surprising given the complex etiology of periodontal disease and the fact that studies of periodontitis in ancient populations pose some technical challenges [45]. Future studies with larger sample sizes,

including both periodontal-positive and periodontal-negative individuals, are needed to determine the microbial association with the disease in postmedieval Latvian individuals.

The analysis of taxonomic results showed that dental calculus samples tended to cluster together, and dental plaque samples formed a separate cluster. In a recent study, it was suggested that a microbial profile gap appears between dental plaque and dental calculus due to the processes of microbial biofilm maturation [8]. Here, when comparing postmedieval dental calculus samples to modern dental plaque and calculus samples within the same geographical location, the results indicate that biofilm type can have a greater impact on microbial communities than chronological origin of the sample (historic vs. modern). Although we can locate potentially pathogenic bacteria on the map of postmedieval dental calculus from Latvia, additional studies are required to reveal paths of microbial coexistence and disease-specific microbial profiles in dental calculus ecosystems.

There are several limitations in our study. First, the limited historical calculus sample size could have prevented the detection of the differences in microbiome composition. Tooth type is known to be a factor influencing plaque/calculus microbial communities [46,47]; however, we were not able to control for the tooth type used in our study. Additionally, the possible lifestyles of the individuals was determined based on the burial place and historical evidence of burial practices in postmedieval Latvia and thus does not include all possibilities of lifestyle/dietary/health changes during the lifetime.

5. Conclusions

Overall, the results showed good preservation of ancient DNA in historical dental calculus samples, providing reliable insight into the composition of the postmedieval oral microbiome of Latvian individuals, and the relative stability of the classifiable oral microbiome composition was observed. Significant differences between microbiome profiles of dental calculus and dental plaque samples were identified, suggesting microbial adaptation to a specific human body environment.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4425/12/2/309/s1, Figure S1: Application of SourceTracker methodology to identify the percentage contribution of each potential source to the microbiome of historic dental calculus samples using a Bayesian model. Figure S2: Analysis of bacterial profiles in blank and burial soil samples: (**a**) Species-level composition of laboratory blank samples; (**b**) Species-level composition of burial soil samples. The most abundant 30 species are shown. Figure S3: Damage plots generated from sequencing reads obtained from 16th–17th century human archaeological dental calculus samples demonstrated a pattern characteristic of ancient DNA. Reads were mapped to the reference genome of *Actinomyces* sp. oral taxon 414. Representative plots from three historic calculus samples of the C > T and G > A nucleotide transition frequencies at the 5′ and 3′ ends of DNA fragments, respectively, are shown. Figure S4: Damage plots generated from sequencing reads obtained from 16th–17th century human archaeological dental calculus samples demonstrated a pattern characteristic of ancient DNA. Reads were mapped to the reference genome of *Olsenella* sp. oral taxon 807. Representative plots from three historic calculus samples of the C > T and G > A nucleotide transition frequencies at the 5′ and 3′ ends of DNA fragments, respectively, are shown. Figure S5: Shannon diversity analysis and permutational multivariate analysis of variance of bacterial profiles in 16th–17th century human archaeological dental calculus based on three different variables: sex, possible lifestyle and age group. *p* values are shown. Figure S6: Shannon diversity analysis and permutational multivariate analysis of variance of bacterial profiles in 16th–17th century human archaeological dental calculus based on two different variables: tooth type and presence/absence of caries. *p* values are shown. Table S1: Sequenced sample data. Table S2: The relative abundance of bacterial species in ancient calculus, modern calculus and modern plaque and burial soil samples. The 100 most abundant species are shown. Table S3: The relative abundance of bacterial species in burial soil, ancient calculus, modern calculus, and modern plaque samples. The 100 most abundant burial soil species are shown.

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Institutional Review Board Statement: The study of modern oral samples was reviewed and approved by Riga Stradins University Research Ethics Committee, decision no. 6-3/4/5 (25.04.2019). No permissions were required for the historical samples. The excavated skeletal material is curated at the Institute of Latvian History, University of Latvia. The material is accessible for research by prior arrangement in accordance with the Institute's regulation No 2015/253.

Informed Consent Statement: Before the collection of modern dental calculus and plaque samples, informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All raw historic calculus DNA sequencing data are publicly available at the ENA under accession PRJEB40382.

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III. Exploring DNA preservation and taxonomic diversity in postmedieval human tooth samples in Latvia.

Highlights:

Part of the results from this publication was used in these theses.

- The preservation of aDNA in human archaeological tooth samples was studied.
- Human and oral microbial DNA was more fragmented than environmental DNA.
- Microbiome profiles were similar for the short length and total DNA fractions.

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Exploring DNA preservation and taxonomic diversity in postmedieval human tooth samples in Latvia

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ABSTRACT

Ancient DNA (aDNA) provides unique opportunities to explore various evolutionary and ecological processes. In particular, aDNA studies offer new possibilities for the in-depth investigation of historical social structures at local and regional scales, reconstruction of ancient human microbiomes, and identification of ancient pathogens. The success of these studies depends on the preservation of aDNA, as well as on our ability to distinguish between historical DNA fragments, either human, animal, plant or microbial, and contaminant modern environmental DNA. The aim of this study was to examine the preservation of aDNA in human postmedieval archaeological tooth samples in Latvia dated 15th–17th century CE. The taxonomic composition of the short DNA fragment fractions was compared to that of the total DNA samples to explore the presence and diversity of environmental bacteria in aDNA datasets.

Samples were analyzed by a shotgun metagenomics-based approach. The taxonomic profiles of metagenomics data revealed that the majority of microbial phyla/genera/species belonged to the typical soil microbiota, indicating the contamination of archaeological samples by environmental microorganisms. No significant differences in alpha or beta diversity indices were found between the short and total DNA fractions. The presence of soil microbiota in the short DNA fractions and the discordance between the microbial patterns suggested the fragmentation of the historical environmental DNA. The proportions of both human and oral microbial DNA were significantly higher in the short DNA fragment libraries than in the total DNA samples, and DNA reads showed characteristic damage patterns, suggesting severe degradation of endogenous aDNA. The human aDNA yield obtained in this study was sufficient to perform molecular sex typing, and the genetic results confirmed morphological data in all cases.

Overall, sufficient preservation of endogenous aDNA was detected in human archaeological tooth samples in Latvia dated 15th–17th century CE, which enables future studies on early-modern populations and pre-industrial oral microbial communities. The analysis of total DNA samples along with short DNA fragments may provide additional information, such as the microbial composition of the burial environment. Concomitant analysis of historical and modern environmental DNA could provide additional data for deciphering the complete ancient microbiome profiles.

1. Introduction

Ancient DNA (aDNA) provides unique opportunities to explore evolutionary and ecological processes such as the history of natural selection and diversity, relationships in paleoecological systems, and interactions between pathogens and their hosts (Orlando and Cooper, 2014; Skoglund and Mathieson, 2018; Warinner et al., 2017). In particular, aDNA studies offer new possibilities for the in-depth investigation of historical social structures at local and regional scales, and aDNA studies have helped to decode human history on the Eurasian continent (Översti et al., 2019). However, retrieving viable aDNA from archaeological sources such as skeletal remains (bone and teeth) or secondary substrates (dental calculus and paleofeces) is challenged by several factors that compromise the molecular stability and quality of

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DNA (Margaryan et al., 2017). DNA molecules have the ability to survive for thousands of years under favorable conditions; at the same time, they are very fragile and are known to undergo fragmentation processes that begin shortly after death (Dabney et al., 2013). Different rates of DNA fragmentation have been observed for different geographic areas and tissue types (Adler et al., 2011). Whole teeth or tooth roots are widely used in aDNA studies because DNA is protected from many environments by the hard enamel and cementum layers (Adler et al., 2011; Melchior et al., 2008), and it was reported that levels of endogenous DNA in well-preserved ancient teeth can often be comparable to those isolated from human petrous bone (Hansen et al., 2017). While the analysis of human DNA is the utmost goal of many aDNA studies, characterization of metagenomic aDNA in tooth and dental calculus samples provides additional important information, such as natural constituents of the human microbiota in the past and the identification of ancient pathogens and infectious diseases (Warinner et al., 2017). Several studies have reported ancient pathogens detected in teeth, as dental pulp is directly exposed to blood-borne pathogens (Drancourt et al., 1998; Drancourt et al., 2005; Rasmussen et al., 2015). The chance of finding an ancient pathogen in a tooth sample is considered to be much higher than when using petrous bone from the same individual, as was exemplified by the reproducible detection of *Yersinia pestis* DNA in tooth samples of several human skeletons dated to the Bronze Age and Iron Age (Margaryan et al., 2018). Additionally, traces of plant and animal DNA were detected in ancient dental calculus samples, thus providing insight into lifestyle and food diversity in past centuries (Sawafuji et al., 2020; Warinner et al., 2014; Weyrich et al., 2017). However, the presence of environmental contamination heavily influences the deciphering of complete ancient microbiome profiles and the identification of specific ancient pathogen DNA in skeletal elements (Rasmussen et al., 2015). In addition to the obvious influential characteristics, such as divergence in metagenomic composition between and within individuals, different geographical locations and burial environments, other important factors could be involved, including the storage conditions and the preservation status of the specimens.

Populations in Western Eurasia were subject to various episodes of expansion, population replacement, and admixture between divergent groups (Slatkin and Racimo, 2016). Latvians are a Baltic-speaking population of Northern Europe, and historic genomes and aDNA studies could help to investigate several issues related to Latvian ethnogenesis, such as a history of population transformations and diversity, differentiation of regional subpopulations, migration events and genetic admixture with neighboring populations. The aim of this study was to examine the preservation of aDNA in human postmedieval archaeological tooth samples in Latvia dated 15th–17th century CE. The taxonomic composition of the short DNA fragment fractions was compared to the total DNA samples to explore the presence and diversity of environmental bacteria in aDNA datasets.

2. Materials and methods

2.1. Characteristics of archaeological tooth samples

Archaeological human tooth samples, dated between the 15th and 17th centuries AD, were collected from three cemeteries in Riga, Latvia: St. Gertrude's Church cemetery (samples T2, T3, T9); the Dome Square cemetery (sample TZA3), and St. Peter's Church cemetery (sample TZA4) (Table 1). St. Peter's Church cemetery and the Dome Square cemetery are located in the Old Riga District, whereas St. Gertrude's Church cemetery is located outside the Old Riga medieval city wall and has been associated with St. Gertrude's medieval village.

Prior to tooth sample collection, archaeological skeletons were inspected for the presence of any disease-specific signs such as degenerative bone changes, tumors and abnormalities associated with infectious diseases (Pinhasi and Mays, 2008). Mouth cavities were inspected for the presence of oral disease lesions and dental calculus. Four skeletons exhibited specific and nonspecific bone lesions, and for one skeleton, no signs of diseases were observable (Table 1). The burial period of the samples was determined by the stratigraphy method, which was used together with the evaluation of archaeological finds (Spirģis, 2012; Tilko, 1998). The approximate age at death was determined by evaluating degenerative changes in the pubic symphysis and using the auricular surface standard method (Brooks and Suchey, 1990; Buckberry and Chamberlain, 2002). Sex was estimated by an experienced anthropologist using pelvic and cranial criteria (Ascádi and Nemeskéri, 1970; Phenice, 1969).

2.2. DNA isolation

During the work with archaeological samples, special care was taken to avoid DNA contamination. To fulfill the safety criteria of aDNA handling and minimize the risk of contamination, specific guidelines developed for aDNA research were followed (Donoghue, 2008; Fulton and Shapiro, 2019). All the surfaces and instruments were cleaned and prepared for aDNA experiments according to rigorous protocols, including cleaning with 5% NaOCl solution and irradiation with UV light (Kazarina et al., 2019). Facility workers were equipped with disposable surgical facemasks, full-body suits and gloves, and only one tooth sample at a time was processed to avoid cross-contamination.

Archaeological material was prepared for aDNA isolation in a

Table 1
Characteristics of samples.

Sample ID	T2	T3	T9	TZA3	TZA4
Cemetery	St. Gertrude's Church	St. Gertrude's Church	St. Gertrude's Church	Dome Square	St. Peter's Church
Century, AD	17th	17th	17th	16-17th	15-16th
Year of excavation	2006	2006	2006	1986	2004
Depth of the grave/sample taken (cm)	320	320	280	150	270
Additional burial above	yes	yes	yes	yes	yes
Fossil skeleton type	Disarticulated skeleton	Articulated skeleton	Articulated skeleton	Articulated skeleton	Articulated skeleton
Sample type	Tooth (molar)	Tooth (premolar)	Tooth (molar)	Tooth (incisor)	Tooth (incisor)
Dental calculus	Yes	No	No	Yes	Yes
Possible diseases	Fusion of the spine, scoliosis, tooth root abscess	Fusion of the spine with local inflammation	Fusion of the spine	Arthritis, periostitis	–
Age (years)	30–35	20–25	16–18	45–50	30–35
Sex	female	male	unknown	female	male
DNA concentration (ng/μl)	1.5	0.91	1.7	3.5	3.8
Mean DNA fragment length (bp)	2705	5058	4449	549	432
Median DNA fragment length (bp)	3200	7000	5500	4000	1600

specially designated separated area of aDNA research facilities. A well-preserved tooth was sampled for each individual. The surfaces of the teeth were abraded with single-use scalpel equipment to remove the calculus when present, and DNA extraction was performed as described previously (Kazarina et al, 2019; Keyser-Tracqui and Ludes, 2004). Briefly, tooth samples were rinsed in bleach (5% solution for 30 sec), rinsed with nuclease-free water and exposed to UV light for 30 min on each side. Samples were then left to dry overnight at room temperature. The next day, whole tooth samples were reduced to fine powder using a CryoMill (RETSCH, Germany). Sample decalcification was performed by incubation of 1 g of powder in extraction buffer (5 mM ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 8.0), 0.3 M sodium acetate, and 1 mg proteinase-K/mL) overnight at 50 °C with continuous vertical rotation. DNA was separated from cellular debris by phenol/chloroform/isoamyl alcohol (25/24/1, v/v) extraction. Purification and concentration of DNA samples were performed using the Genomic DNA Clean & Concentrator Kit (Zymo Research, United States) following the manufacturer's instructions. The DNA concentration was estimated using a Qubit dsDNA HS Assay Kit (Life Technologies, United States), and the assessment of DNA quality and fragment length was performed using an Agilent High Sensitivity DNA Kit (Agilent Technologies, United States). To control laboratory contamination, DNA isolation procedures were accompanied by corresponding blank samples, which were also treated equally and underwent the same DNA isolation steps using the same DNA purification and concentration reagents.

2.3. Library preparation and sequencing

Metagenomics sequencing libraries were prepared using the Ion Plus Fragment Library Kit (Ion Torrent™, Thermo Fisher Scientific, USA) following the manufacturer's instructions. Each DNA sample was divided into two aliquots of 10 μl each, which were further used to prepare two types of libraries: non-fragmented and fragmented libraries. For non-fragmented libraries, with the intention of keeping only short DNA fragments (<350 bp), aliquots underwent size selection using NucleoMag® NGS Clean-up and Size Select magnetic beads (Macherey-Nagel, USA). In this study, these libraries were named "Short DNA library". For fragmented libraries, with the intention of analyzing the total DNA in the samples, including long DNA fragments, prior to specific barcode attachment, aliquots underwent enzymatic fragmentation with an Ion Shear™ Plus Reagent Kit (Ion Torrent™, Thermo Fisher Scientific, USA) following the manufacturer's instructions. In this study, these libraries were named "Total DNA library". Further library preparation steps were identical for both library types and, according to the manufacturer's instructions, involved the following steps: specific barcode ligation, library amplification and assessment of library quality on the Bioanalyzer 2100 instrument with an Agilent High Sensitivity DNA Kit (Agilent Technologies, USA). Sequencing was performed on an Ion Proton™ System (Thermo Fisher Scientific, USA).

2.4. Sequencing data analysis

Sequencing data were preprocessed on the local Ion Torrent Proton server, assigning data to each sample and removing barcodes and adapters. The resulting data were exported from the server in the format of BAM files, which further underwent a quality control workflow and removal of two overrepresented remaining adapter sequences using analysis tools on the Galaxy public server (Goecks et al., 2010; Kosakovsky Pond et al., 2009). Sequences that underwent quality filtering and reads with quality PHRES scores <20 were excluded from further manipulations. Kraken2 v2.0.7 with the use of the standard Kraken2 database (Wood and Salzberg, 2014) was used to assign taxonomic labels to the resultant metagenomic DNA sequences. Kraken taxonomy report files were further manipulated with the use of the Pavian web application – taxonomy quality assignment was generated (Breitwieser et al., 2020). Contamination control was performed using the R package "Decontam" (Davis et al., 2018; Salter et al., 2014).

Statistical analyses were performed and visualized using the MicrobiomeAnalyst public server (https://www.microbiomeanalyst.ca/) (Chong et al., 2020; Dhariwal et al., 2017) and Calypso public server (https://cgenome.net/calypso/) (Zakrzewski et al., 2017). Community alpha diversity was estimated by the Shannon diversity index, which evaluates both the richness and evenness of taxa within the samples. Differences in beta diversity between sample clusters were calculated using analysis of similarities (ANOSIM) and a Bray-Curtis dissimilarity matrix and displayed by principal component analysis (PCA).

2.5. Identification of human DNA

To identify the presence of human DNA, sequencing reads obtained from tooth samples were processed *in silico* with the Efficient Ancient Genome Reconstruction pipeline (EAGER, v.1.92) (Peltzer et al., 2016). De-multiplexed, adapter-clipped reads were aligned to a human genome reference (GRCh38, GCA_000001405.28) with BWA (Li and Durbin, 2009). DamageProfiler was used to characterize DNA damage (Neukamm et al., 2020). For the characterization of genomic data, the PALEOMIX pipeline was used (http://geogenetics.ku.dk/publications/paleomix) (Schubert et al., 2014).

2.6. Preservation of oral microbial species DNA

Preservation of oral microbial DNA in historic tooth samples was assessed. The metagenomics sequencing read files of historic tooth samples were analyzed using MALT 0.5.0 (Herbig et al., 2017) (https://software-ab.informatik.uni-tuebingen.de/download/malt/welcome.html), using all complete bacterial genomes available from NCBI Assembly in August 2020 as a reference. Bacterial reads were extracted using SAMtools, mapped to the reference genomes of the oral microorganisms *Olsenella* sp. oral taxon 807 and *Streptococcus sanguinis*, and DNA deamination rates were calculated using MapDamage (Jónsson et al., 2013) within the EAGER pipeline.

3. Results

3.1. Pre-sequencing DNA data

The overall results of DNA fragment length distribution analysis and total DNA concentration for the tooth samples used in this study are presented in Table 1 and Supplementary figure 1. The total DNA concentrations of the tooth samples ranged from 0.91 to 3.8 ng/μl. Samples showed a wide range of DNA length distributions, with mean DNA fragment lengths ranging from 432 to 4449 bp. The shortest mean and median DNA fragment lengths were observed for the oldest sample, TZA4, which was dated between the 15th and 16th centuries AD, while all three samples dated to the 17th century CE showed much greater mean DNA fragment length (Table 1). Based on these data, both short DNA and total DNA libraries were prepared for all samples for sequencing purposes.

3.2. Sequencing data

The total library concentration and the number of raw sequencing reads obtained are presented in Table 2. The sequencing reads were deposited in the European Nucleotide Archive (ENA) under study number PRJEB47251. For the short DNA fragment fractions (i.e., non-fragmented libraries), library concentrations for the tooth samples ranged from 0.09 ng/μl to 1.43 ng/μl, and for the total DNA libraries (i.e., DNA was additionally fragmented prior to the analysis), the concentration range was 0.347–3.07 ng/μl. After sequencing, on average, 2.9 million raw reads were obtained per sample (ranging from 0.4 to 8.5 million reads). Following data preprocessing, which implemented

Table 2
Sequencing data.

Sample ID	Library type*	Library concentration, ng/μl	Reads obtained	Median read length, bp	Reads mapped to human genome, No (%)	Sex assigned by sequence analysis/complementary to morphological characteristics
T2	Short	0.248	368,870	122	57,518 (15.6)	XX/yes
T2	Total	1.050	7,946,030	140	195,991 (2.5)	
T3	Short	0.219	195,972	151	23,304 (11.9)	XY/yes
T3	Total	0.347	4,122,576	101	129,687 (3.1)	
T9	Short	0.090	710,005	145	25,276 (3.6)	XY/n.a.
T9	Total	3.070	8,449,132	123	11,861 (0.1)	
TZA3	Short	0.819	3,385,668	156	148,768 (4.4)	XX/yes
TZA3	Total	0.605	3,827,287	127	59,387 (1.6)	
TZA4	Short	1.430	3,331,062	157	278,886 (8.4)	XY/yes
TZA4	Total	1.150	4,290,308	129	130,133 (3.0)	

* Short: non-fragmented sequencing library, only short DNA fragments were included (<350 bp). Total: sequencing library was prepared by total DNA fragmentation including long DNA fragments.

duplicate and adapter removal, as well as quality filtering, taxonomic analysis of sequenced reads was performed. After removing contaminants, data on the remaining taxa were further analyzed for all samples. Reads that could not be classified at the phylum or genus levels were omitted.

3.3. Taxonomic analysis

3.3.1. Phylum level analysis

Metagenomic analysis of the sequencing data of archaeological tooth samples showed that the most represented phyla with prevalence >1% were Actinobacteria, Proteobacteria, Chordata, Firmicutes, Chloroflexi, Bacteroidetes and Planctomycetes. These phyla were the most dominant in both the short and total DNA fractions, and other phyla accounted for <2.3% of the sequencing reads. The most represented phyla in short DNA fractions isolated from tooth samples were Proteobacteria (30.6%), Actinobacteria (28.74%) and Chordata (27.83%), followed by less abundant Firmicutes (5.56%), Chloroflexi (2.53%), Bacteroidetes (1.67%), Planctomycetes (1.02%), Acidobacteria (0.31%), Cyanobacteria (0.19%) and Fusobacteria (0.19%) (Fig. 1A). Only slightly different phyla patterns were observed for the total DNA samples isolated from the same archaeological tooth samples: the most represented phyla were Proteobacteria (35.1%) and Actinobacteria (38.87%), followed by Firmicutes (10.23%), Chordata (8.77%), Planctomycetes (1.76%),

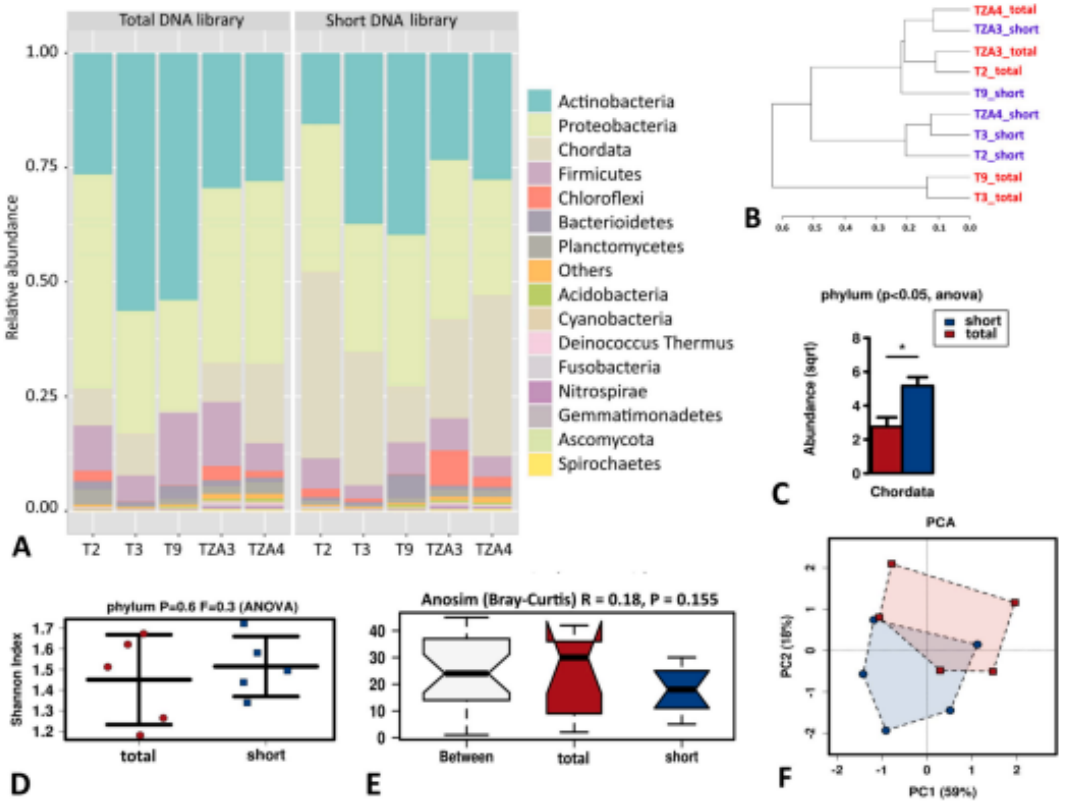

Fig. 1. Phylum-level analysis of 15th-17th century human archaeological tooth samples. A. Stacked plots of taxonomic classification at the phylum level. The relative abundances of the most abundant phyla are shown. B. Clustering dendrogram. The red color represents the total DNA samples, and the blue color represents the short DNA fragment fractions. Samples with relatively similar phylum profiles were clustered closer together. C. Comparison of the relative abundances of the phyla between short and long DNA fractions. Asterisks denote significant differences ($P < 0.05$) between groups. D. Shannon diversity analysis. *P* values are indicated. E. Beta diversity analysis. *P* values are indicated. E. Principal component analysis (PCA) plots of the short and total DNA fractions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Bacteroidetes (1.62%), Chloroflexi (1.4%), Acidobacteria (0.32%), Cyanobacteria (0.27%) and Fusobacteria (0.18%) (Fig. 1A). The only phylum that showed a statistically significant difference in terms of mean relative abundance was Chordata ($P < 0.05$); this phylum was significantly more abundant in the short DNA fragment fractions of the archaeological tooth samples than in the total DNA (Fig. 1C).

The alpha diversity of the taxonomic composition at the phylum level did not show a significant difference between short DNA fractions and total DNA ($P > 0.05$; Fig. 1D). Additionally, ANOSIM did not show a statistically significant difference ($P > 0.05$) in beta diversity between these two sample groups (Fig. 1E), and PCA grouped short and total DNA fractions in two overlapping clusters (Fig. 1F).

3.3.2. *Genus level analysis*

Next, taxonomic patterns of the paired short and total DNA fragment fractions obtained from the archaeological tooth samples were compared at the genus level. In total, 1727 OTUs were identified, and 197 OTUs were removed based on the low abundance filter. Among the remaining 1367 OTUs, the most represented genera in both the short and total DNA fragment fractions were *Homo* (31.78% and 10.43%, respectively) and the soil bacteria *Micromonospora* (11.66% and 20.01%, respectively); these genera were followed by others, which included mostly soil-related bacteria, with average abundance of <5% each (Fig. 2A). However, great diversity between individual samples was observed regarding the most abundant genera: the proportion of reads belonging to the *Homo* genus in tooth samples ranged from 13.65 to 45.9% and from 0.68 to 20.9% for the short and total DNA fragments, respectively. Similarly, the *Micromonospora* genus fraction represented 0.5–31.47% and 0.92–45.55% of the DNA reads in the short and total DNA libraries, respectively (Fig. 2A). Among the ten most abundant genera in short DNA fractions were *Actinomyces* (3.94%), *Streptomyces* (3.07%), *Mesorhizobium* (1.96%), *Olsenella* (1.81%), *Bradyrhizobium* (1.63%), *Pseudomonas* (1.31%), *Streptococcus* (1.12%), and *Pseudorhodoplanes* (0.94%), while those among the total DNA samples were *Streptomyces* (4.32%), *Pseudorhodoplanes* (2.99%), *Brevibacillus* (2.13%), *Actinomyces* (1.98%), *Bradyrhizobium* (1.88%), *Mesorhizobium* (1.83%), *Pseudomonas* (1.58%), and *Clostridium* (1.42%) (Fig. 2A).

Statistical significance was reached for only two genera: the genus *Homo* was significantly more abundant in short DNA fragment fractions than in total DNA ($P < 0.05$), while the genus *Bacillus* was significantly more abundant in total DNA libraries (0.68% vs. 0.24%, $P < 0.05$) (Fig. 2C). There was no statistically significant difference in alpha diversity between short and total DNA fragments ($P > 0.05$; Fig. 2D). Similarly, ANOSIM results did not indicate significant differences in beta diversity between the genus profiles detected within each group of samples ($P > 0.05$; Fig. 2E). Similar results were obtained by PCA, which grouped the samples into two overlapping clusters (Fig. 2F).

3.3.3. *Taxonomical analysis of microbial profiles at the species level*

In total, 4023 OTUs were identified; 763 OTUs were removed based on the low abundance filter, and 3260 features remained after data filtering within all 10 sequenced libraries. During the metagenomics analysis of the sequencing data at the species level, no significant differences in taxa richness or alpha ($P > 0.05$) or beta diversity indices (P

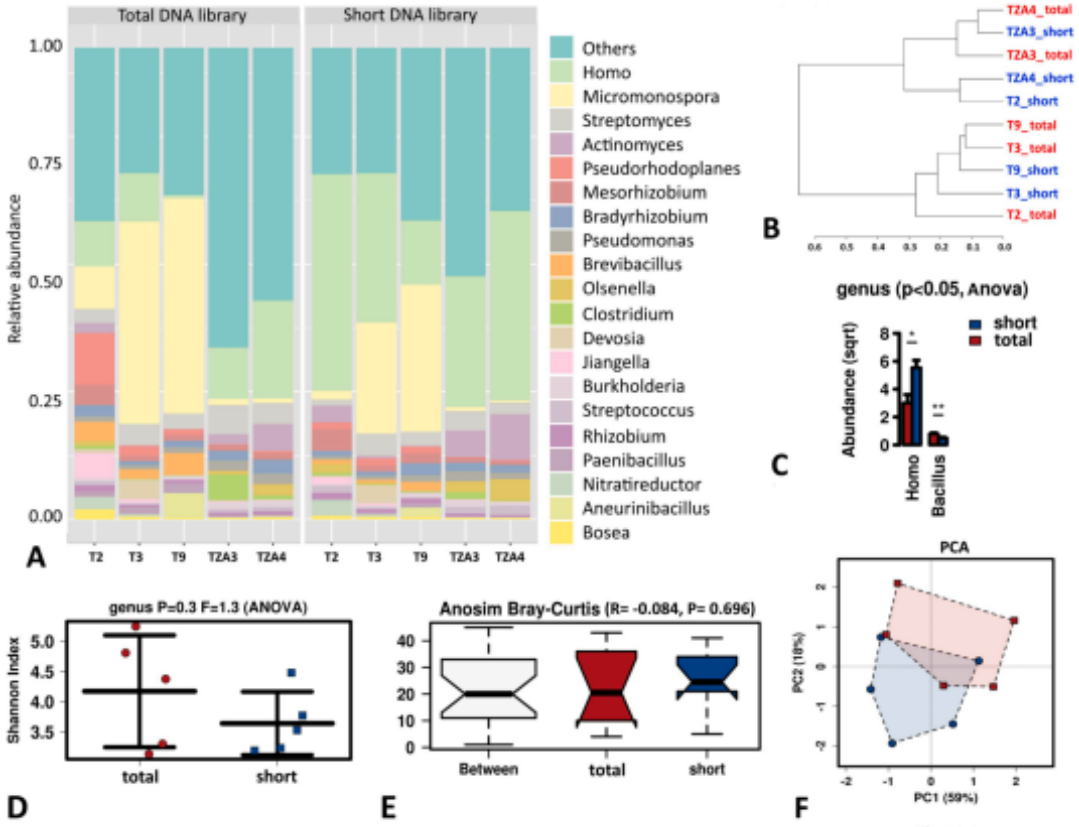

Fig. 2. Genus-level analysis of 15th-17th century human archaeological tooth samples. A. Stacked plots of the taxonomic classification at the genus level. The relative abundances of the most abundant genera are shown. B. Clustering dendrogram. The red color represents the total DNA samples, and the blue color represents the short DNA fragment fractions. Samples with more similar genus profiles were clustered closer together. C. Comparison of the relative abundances of the genera between short and total DNA fractions. Asterisks denote significant differences ($P < 0.05$) between groups. D. Shannon diversity analysis. P values are indicated. E. Beta diversity analysis. P values are indicated. E. Principal component analysis (PCA) plots of the short and total DNA fractions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

> 0.05) were found between the short and total DNA fractions (Fig. 3 B, C). However, the results indicated a discordance between the microbial patterns. Among the ten most abundant bacterial species in the total DNA samples, all but two were soil-related: *Pseudorhodoplanes sinuspersici* (5.89%), *Micromonospora viridifaciens* (5.61%), *Micromonospora narathiwatensis* (4.08%), *Aneurinibacillus* sp. XH2 (2.31%), *Micromonospora inositola* (2.00%), *Micromonospora auratinigra* (1.92%), *Brevibacillus* sp. SCSIO_07484 (1.88%), and *Jiangella* sp. DSM_45060 (1.84%); the two oral microbiome-related bacterial species were *Anaerolineaceae* bacterium oral taxon 439 (1.94%) and *Actinomyces* sp. oral taxon 414 (1.48%) (Fig. 3A, Supplementary Table 1). In contrast, six of the ten most abundant bacterial species within the short DNA fractions belonged to the oral microbial community: *Anaerolineaceae* bacterium oral taxon 439 (8.44%), *Actinomyces* sp. oral taxon 414 (8.04%), *Olsenella* sp. oral taxon 807 (4.84%), *Pseudopropionibacterium propionicum* (2.26%), *Streptococcus sanguinis* (1.08%), and *Desulfobulbus oralis* (0.78%); the four environment-related bacteria were *Micromonospora viridifaciens* (1.47%), *Betaproteobacteria* bacterium GR16_43 (1.14%), *Micromonospora narathiwatensis* (1.05%), and *Pseudorhodoplanes sinuspersici* (0.75%).

For the authentication of oral microbiome preservation in historic tooth samples, the DNA damage patterns of two of the oral microorganisms, *Olsenella* sp. oral taxon 807 and *Streptococcus sanguinis*, were evaluated. The analysis revealed signs of cytosine to thymine substitutions at the ends of DNA fragments characteristic of aDNA, but only for the short DNA fractions (Supplementary figure 2).

3.4. Human DNA analysis

Raw sequenced reads were mapped to the human reference genome GRCh38 using BWA as implemented in the EAGER pipeline. For the archaeological tooth samples, on average, 8.78% (3.6–15.6%) of the short DNA reads aligned to the reference. MapDamage analysis of these reads revealed distinctive cytosine-to-thymine damage patterns that are characteristic of aDNA (Table 2, Fig. 4). In comparison, 1.66% (0.1–3.1%) of the total DNA library reads aligned to the reference and DNA damage patterns were not observed (Table 2, Fig. 4).

Sex of the human remains was determined based on calculations using the ratio of X chromosome to mean autosome coverage and Y chromosome to mean autosome coverage; only sequencing data generated from the short DNA libraries were used (Table 2). The results for samples T2, T3, TZA3 and TZA4 matched the sex of the individuals, which was determined based on morphological identification (the sex of individual T9 was unknown).

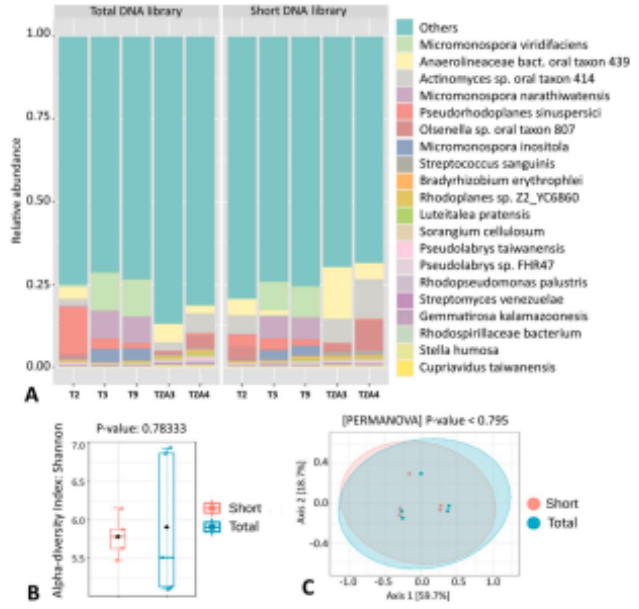

Fig. 3. Species-level taxonomic analysis of microbial profiles in 15th-17th century human archaeological tooth samples. A. Stacked plots of the taxonomic classification at the species level. The relative abundances of the most abundant microbial species are shown. B. Shannon diversity analysis. *P* values are indicated. C. Principal coordinates analysis (PCoA) derived from Bray–Curtis distances among samples of the four groups ($p < 0.001$ by PERMANOVA). For each axis, the percent of variation explained was reported in square brackets.

4. Discussion

Historic genomes and metagenomes can provide valuable information on past populations, including plants, animals and humans, as well as ancient microbial communities. The success of these studies depends on the preservation of aDNA, as it is the most limiting factor in ancient genomic research (Damgaard et al., 2015). It is known that the vast majority of human remains contain low percentages of endogenous molecules, with most comprising only 1% of the library (Key et al., 2017). However, endogenous human DNA content can vary significantly based on taphonomic changes and source material (Dabney et al., 2013; Hansen et al., 2017). Temperate and Arctic regions have yielded many more aDNA sequences than tropical regions, partly because conditions are more favorable to the preservation of aDNA (Slatkin and Racimo, 2016). The average annual air temperature in Latvia, a country in the Baltic region of Northern Europe, is + 5.9 °C (Latvian Environment, Geology and Meteorology Centre, https://www.meteo.lv/en/). Such climate conditions with fairly severe winters, when the daytime temperatures are usually below zero, could favor the preservation of DNA in burial environments. Indeed, in our study, the average endogenous content of human aDNA in tooth samples from archaeological human remains dated 15th-17th century CE was 8.78%. These results exhibit good agreement with those reported for tooth samples dated to the 18th century from Denmark (i.e., 2.09–15.9%; Damgaard et al, 2015) and indicate good preservation of human aDNA in tooth samples of human remains buried for approximately four to five centuries in the temperate climate zone. The aDNA yield obtained in this study was sufficient to perform molecular sex typing, and the genetic results confirmed morphological data in all cases. Such access to aDNA data and histotic genomes will be highly important in future studies aimed at deciphering histories of early modern-age Latvians (16th–18th century) that are not retrievable from contemporary individuals, and could explain the contemporary genetic diversity in Latvia. For example, as recently demonstrated by a study of human mitochondrial DNA lineages in Iron-Age Fennoscandia (Översti et al., 2019), aDNA research provided information about the genetic shift from hunter-gatherers towards farmers in North-East Europe.

The first systematic studies of aDNA properties back in 1989 revealed that DNA extracted from 4- to 13,000-year-old dry remains of soft tissues consists of 40–500-bp-long fragments (Paabo, 1989). By studying ancient bones of the extinct New Zealand moa, the average DNA half-life was calculated to be 521 years for a 242 bp segment of mitochondrial DNA (Allentoft et al., 2012). Usually, DNA fragmentation to <100 base pairs is observed in ancient samples, and the short length of DNA molecules is widely used as a measure of authenticity of aDNA (Key et al., 2017). On the other hand, it is known that bacterial DNA degradation follows different paths in comparison to host DNA (Key et al., 2017, Schuenemann et al., 2013). Therefore, bacterial DNA, compared to the host DNA strands, may appear to be longer and less damaged by hydrolytic DNA deamination, thus resembling exogenous sample contamination. In addition, higher rates of DNA damage were reported in samples from warm, humid environments than in samples from Europe (Adler et al., 2011). However, the results of a recent study have shown that some well-preserved paleofeces from the southwestern U.S. and Mexico contain longer (average mode length = 174 bp) aDNA

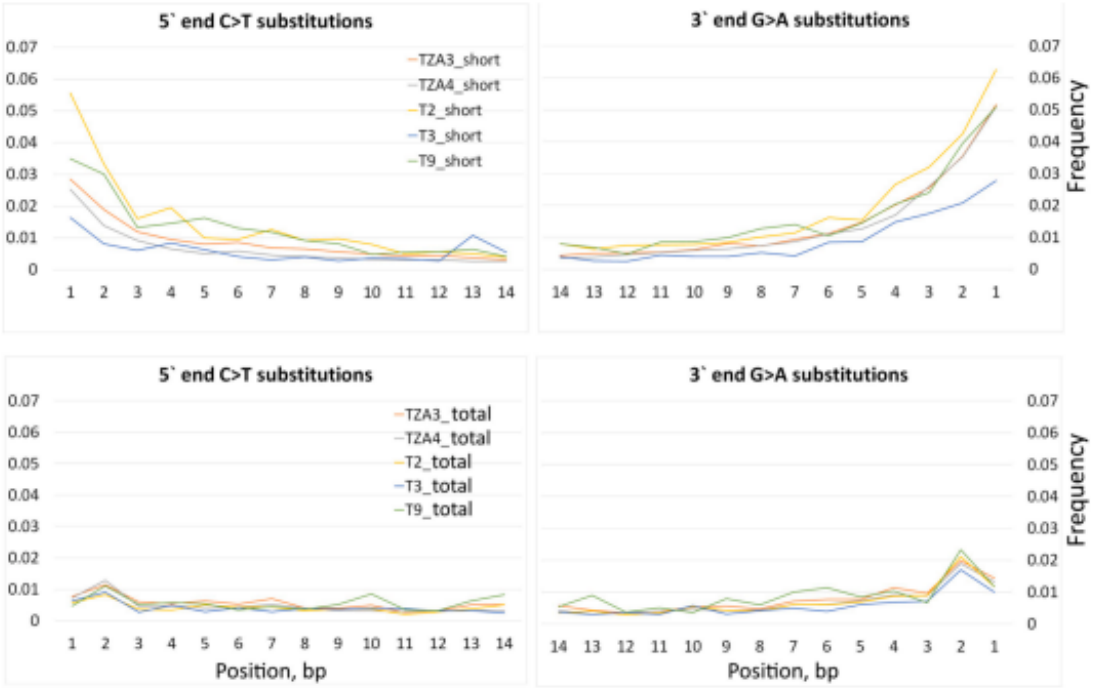

Fig. 4. Damage plots generated from sequencing reads obtained from short and total DNA fractions in archaeological tooth samples. Reads were mapped to a human reference genome. The reads obtained from short DNA libraries demonstrated a pattern characteristic of ancient DNA.

fragments (Wibowo et al., 2021).

Within our study, the mean fragment length of the total DNA extracted from human postmedieval archaeological tooth samples from Latvia dated 15th–17th century CE was approximately 433 to 4449 bp, indicating the presence of modern environmental contamination. Thus, the taxonomic profiles of metagenomics data were analyzed for the paired short DNA fractions (median length 122–157 bp), which were supposed to contain aDNA molecules, and total DNA samples, which were fragmented prior to the analysis to fit the sequencing technologies used in our study. Based on the alpha and beta diversity analysis at the phylum, genus and species levels, clear separation between short and total DNA fragment fractions was not observed, and the majority of microbial phyla/genera/species belonging to the typical soil microbiota confirmed the contamination of archaeological samples by environmental microorganisms. After inhumation, the human body, including teeth, is inevitably exposed to the burial environment, which could explain the high load and diversity of the typical soil microbiome observed in archaeological samples. Additionally, in our study, the presence of soil microbiota in short DNA fragment fractions indicates that, along with endogenous human aDNA and ancient oral microbiota, old contaminant molecules from the burial environment most likely underwent degradation processes. Environmental DNA (eDNA) is often fragmented to a size of <150 bp (Pedersen et al., 2015). As one example, the study by Sampietro and colleagues showed that the damage profile and rate of old contaminant sequences were similar to those of endogenous DNA sequences (Sampietro et al., 2006). Likewise, eDNA preservation is highly dependent on the conditions of the environment. Under favorable conditions of cold, dry permafrost, eDNA can survive for hundreds of years (Willerslev et al., 2003), whereas in temperate water, it degrades over a period of several weeks (Dejean et al., 2011; Thomsen et al., 2012). Therefore, the DNA fragment size selection strategy could not easily eliminate the impact of the possible presence of eDNA in ancient microbiome datasets.

Unsurprisingly, in this study, the proportions of both human DNA and oral microbial DNA were significantly higher for the short length DNA fragment fractions, suggesting severe degradation of the endogenous aDNA, and the obtained DNA reads showed an excess of cytosine to thymine misincorporations at the 5′ ends, which is indicative of authentic aDNA (Briggs et al., 2007, Brotherton et al., 2007, Key et al., 2017). On the other hand, the proportion of the most abundant soil-related bacterial species, such as *Pseudorhodoplanes sinuspersici*, *Micromonospora viridifaciens*, and *Micromonospora narathiwatensis*, was much larger in the total DNA samples most likely representing modern environmental contamination. This result indicates that sequencing of total DNA samples isolated from archaeological remains along with short aDNA fragments could help to determine the nature of exogenous contamination from the burial environment. However, the abundances of several other environmental bacterial species, including *Bradyrhizobium erythrophlei*, *Rhodopseudomonas palustris*, *Sorangium cellulosum* and *Luteitalea pratensis*, were similar for both DNA library types possibly indicating the presence of historical eDNA fragments in the short DNA fractions. Additional studies are required to decipher whether this result suggests that the rate of degradation for different microorganisms can vary, or instead indicates that humans of the early modern age had increased contact with soil microorganisms during their lifetimes. Additionally, the possibility exists that in some cases, eDNA might be more exposed to degradation factors than the DNA of bacteria trapped inside an archaeological sample, and in this way may be protected from robust environmental exposure.

In our study, DNA fragmentation was performed for the total DNA sample aliquots prior to sequencing; this could explain the absence of typical aDNA damage patterns for human and oral bacterial DNA reads in total DNA libraries. The possibility of modern laboratory DNA contamination in our study was ruled out by rigorous aDNA handling protocols indicating, along with lower yield, that these DNA reads more likely represented short DNA fractions. However, we were not able to prove the exact origins or to calculate the exact lengths of the historical human and oral bacterial DNA reads that were present in the total DNA samples due to the limitations in sequencing technologies. Thus, for sex determination purposes, only authenticated human aDNA reads from the short DNA libraries were used in this study. Similarly, more profound analysis methods are needed to distinguish whether the longer

bacterial DNA fragments in the total DNA libraries are modern strains typical of the soil microbiota, or are historical strains which are well preserved within the archaeological samples and/or have persisted, due to their greater numbers. Nevertheless, as recently demonstrated by a human paleofeces study (Wibowo et al., 2021), preservation of aDNA in archaeological samples is relatively understudied, and more research is needed.

5. Conclusions

Overall, sufficient preservation of endogenous aDNA was detected in human archaeological tooth samples in Latvia dated 15th–17th century CE, which enables future studies on early-modern human populations and pre-industrial oral microbial communities. For ancient microbiome studies, the use of DNA length for aDNA authentication is insufficient. Concomitant analysis of historical and modern eDNA fragments may provide additional important data on environmental microbial contamination, which is important for deciphering the complete ancient microbiome profiles and could provide insight into composition of historical burial environments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jasrep.2021.103213.

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4. Discussion

Recent advantages in sequencing technologies have provided us an opportunity to access our ancestral microbial information. Ancient human-related microbial studies have gone a long way since their dawn in 2008 and thanks to constant technological progress, there is still much more to go. Taken together, these studies widen our notion about historic human lifestyle, health and diseases as well as help us challenge the paradigm of modern public health.

Within this work, I have investigated ancient DNA (aDNA) from several archaeological human remains material types: bones, dental calculus and teeth. All samples were collected from postmedieval burial sites in Latvia. I have evaluated them in terms of historic biomolecule preservation abilities and examined their limiting factors together with their possible contribution to the realm of historic human microbiome studies. I've also attempted to characterize historic human microbiome, based on the information obtained. Each material expressed its unique properties, provided new insights and opened novel questions.

In the first study, several bone samples (vertebrae, rib and skull) were analysed for their potential to serve as a secondary deposit material for historic microorganism DNA. Overall, their microbiome data showed a close correlation with the typical composition of the soil microbiome. However, some exceptions were observed. The most noticeable deviation between bone and soil samples was witnessed in the proportion of the *Firmicutes* phylum, which was significantly more abundant in bone samples than in the corresponding soil samples (P=0.0337). Although *Firmicutes* phylum is common to soil microbiome, its abundance rarely exceeds 10% (Janssen, 2006). In our study, mean relative abundances of *Firmicutes* phylum in soil vs bone samples ranged from 6.3% to 43.9% respectively. This fact is intriguing because *Firmicutes* phylum is known to be dominant in human gut microbiome (Hollister et al., 2014). Also, it is noteworthy to mention that recently *Firmicutes* were found to be most abundant microbial group in the palaeofeces of a pre-Columbian mummy (Santiago-Rodriguez et al., 2015). Further, examining *Firmicutes* phylum to the genus level, among typical soil *Firmicutes* genera we also found *Sporanaerobacter* genus, which was the most abundant *Firmicutes* genus in bone samples. It also correlates to above mentioned Santiago-Rodriguez study, which identified *Sporanaerobacter* in human gut microbiome of the pre-Columbian mummy. Moreover, we know that members of the gastrointestinal bacterial community appear in blood within 24 h post-mortem while being released into the abdominal cavity (Hyde et al., 2013; Yang et al., 2012). *Sporanaerobacter* is also known to be constantly observed in later stages of tissue decomposition under anaerobic conditions (Kim et al., 2017; Yang et al., 2012). All together, these observations hint to the fact that members of authentic microbial communities of an organism may

indeed be introduced to organism's bones during post-mortem processes of soft tissue decomposition and stay trapped inside, thus providing useful material for historic DNA research.

In the second study, microbial composition of dental calculus samples from archaeological human teeth from Latvia was compared to microbial composition of modern Latvian dental plaque and dental calculus samples. Firstly, the results of this study clearly indicated that despite some environmental contamination, historic dental calculus samples provide a reliable snapshot of bacterial oral communities from past individuals. Results also clearly showed that human oral microbiome composition varies within different locations of oral cavity. Recent study by Irina Velsko et al. investigated the question of dental plaque transformation into dental calculus, finding that bacterial communities of the two formations carry significant differences (Velsko et al., 2019). Our study demonstrated comparable results within historical and modern Latvian individuals, indicating that biofilm type can have a greater impact on microbial communities than chronological origin of the sample (historic vs. modern). This is an important note that should be taken into account whenever one is willing to compare modern oral microbiome data to historical data.

Several commensal bacterial species of oral cavity were also detected within historic dental calculus data. These included *Streptococcus sanguinis, Streptococcus cristatus* and *Lautropia mirabilis*. While archaeological samples included in our study were without clear evidence of periodontal disease, the periodontal pathobiont *Desulfobulbus oralis* was also present among 10 most abundant bacterial species from postmedieval dental calculus samples. Nevertheless, given the complex etiology of periodontal disease and the fact that studies of periodontitis in ancient populations pose some technical challenges (Raitapuro-Murray et al., 2014), to fully study this question a larger number of samples from both periodontal-positive and periodontal-negative individuals are needed to determine the microbial association with the disease in postmedieval Latvian individuals.

Strong presence of soil bacterial species was detected in ancient dental calculus specimens as opposed to modern oral samples. This finding can be easily explained by the direct impact of the burial environment. However, industrialization, urbanization, and modern food processing have dramatically reduced human contact with soil microorganisms. There might be a chance that a fraction of environmental bacteria within historic dental calculus samples represents traces of dirt that may be incorporated into dental calculus over a lifetime of eating food that is not fully cleaned. Also, a fraction of environmental bacteria found within samples, could potentially represent members of historic human oral microbiome. To test this hypothesis more studies are needed.

The third research included in my Thesis focuses on questions of aDNA preservation and environmental contamination, comparing taxonomic composition of short DNA fragment fractions to total DNA extracted from human postmedieval archaeological tooth samples from Latvia 15th–17th century AD. Knowing that usual abundance of endogenous molecules within human remains is generally very low, we can conclude that tooth samples showed relatively good average yield of endogenous human aDNA – 8.78%. This could be explained with the fact that the average annual air temperature in Latvia, a country in the Baltic region of Northern Europe, is +5.9°C (Latvian Environment, Geology and Meteorology Centre, https://www.meteo.lv/en/). Such climate conditions with fairly severe winters, when the daytime temperatures are usually below zero, could favour the preservation of DNA in burial environments.

Within this study, the mean fragment length of the total DNA extracted from human postmedieval archaeological tooth samples was approximately 433 to 4,449 bp, indicating the presence of environmental contamination. Thus, the taxonomic profiles of metagenomics data were analysed for the paired short DNA fractions (median length 122-157 bp), which were supposed to contain aDNA molecules, and total DNA samples, which were fragmented prior to the analysis to fit the sequencing technologies used in our study. Based on the alpha and beta diversity analysis at the phylum, genus and species levels, clear separation between short and total DNA fragment fractions was not observed and the majority of microbial phyla/genera/species belonging to the typical soil microbiota confirmed the contamination of archaeological samples by environmental microorganisms. Additionally, in our study, the presence of soil microbiota in short DNA fragment fractions indicates that, along with endogenous human aDNA and ancient oral microbiota, old contaminant molecules from the burial environment most likely underwent degradation processes. Therefore, the DNA fragment size selection strategy could not easily eliminate the impact of the possible presence of eDNA in ancient microbiome datasets.

The proportion of the most abundant soil-related bacterial species, such as *Pseudorhodoplanes sinuspersici, Micromonospora viridifaciens*, and *Micromonospora narathiwatensis,* was much larger in the total DNA samples, which was expected. However, the abundances of several other environmental bacterial species, including *Bradyrhizobium erythrophlei, Rhodopseudomonas palustris, Sorangium cellulosum* and *Luteitalea pratensis*, were similar for both DNA library types. Additional studies are required to decipher whether this result suggests that the rate of degradation for eDNA might differ from aDNA of historic specimen or instead indicates that humans of the early modern age had increased contact with soil microorganisms during their lifetimes.

Within every in this Thesis incorporated study a question arose weather environmental bacteria, found within archaeological samples, represents environmental contamination of the specimen or is it a trace of historic human microbiome which had a closer connection with environment. Indeed, possibility exists that some microbiome elements that we assume to be environmental are in fact members of historical human microbiome community. Why this might be so and what could it mean for us? Soil is a completely different environment with different purposes and functions, comparing with bacterial biotopes of human organism. A legitimate question comes to mind – why should we be expecting to meet its members within human microbial communities? It appears, that answering this question brings to the surface many important topics.

Soil is the richest natural microbial reservoir on Earth (Daniel, 2005; Naylor et al., n.d.). It is estimated, that 1g of average agricultural soil contains around 5.95×10^9 bacterial cells (much of which remains uncultured and unstudied), not to mention other highly important microbial composition elements such as eukaryotic microbes (fungi, protists), archaea and viruses (Aoshima et al., 2006; Gill et al., 2020). Environmental crisis nowadays expands from macro- to micro-levels, influencing soil microbial biodiversity with many anthropogenic factors. Among those, an increasing use of agrochemicals, low biodiversity of agricultural systems and rigorous soil maintenance practices can be named (Dubey et al., 2019; Ng et al., 2021; Peltoniemi et al., 2021). However, list does not end with that. As a result, we see a drop in the diversity of agricultural plant epithites and endophites and an overall diversity drop within soil microbial communities (Chen et al., 2020; Jacoby et al., 2017; Newman, 2019). Intriguingly, it correlates with the rise of lifestyle diseases in western societies and developing countries (Haahtela et al., 2013).

On the other hand, due to current ecological situation, nowadays it might be difficult to find a natural biotope that would be prospering in species diversity. Same relates to human gut microbiome which is known to be undergoing dramatic diversity loss with the modern lifestyle. From hunter-gatherers to modern society, much of human gut's bacterial alpha diversity has been lost. Beta-diversity, however, has increased which means that within one society humans now have more distant microbial profiles (Conteville et al., 2019; Fragiadakis et al., 2019; Schnorr, 2015; Segata, 2015). Analyzing evolutionary aspect of human microbiome, we also know that during the diversification of African apes there are visible steady changes in microbiome composition. Human microbiomes, however, diverged at an accelerated pace as a result of dramatic microbial diversity loss (Moeller et al., 2014).

The highest bacterial and genetic function diversity that has ever been reported in humans was discovered in 2015 in remote Amazonian jungle hunter-gatherer tribe from a Yanomami Amerindian

village (Clemente et al., n.d.). Within the study, Jose C. Clemente et al. analyzed Amerindian gut, skin and mouth microbiomes. Both skin and gut microbiomes expressed an unprecedented microbial diversity. Interestingly, oral microbiome did not differ much from modern US human oral microbiome in terms of alpha-diversity. Study authors explain this finding with the fact that among other possible factors of influence, Amerindians have a strong tradition of cultivating tobacco and are used to its consuming starting from an early age (Oyuela-Caycedo and Kawa, 2015). This resonates with our findings from the second article included in the Thesis: analyzing historic dental calculus samples in comparison to modern dental calculus specimens no significant alpha diversity was observed among specimen groups. Keeping in mind the fact that postmedieval Latvia of 16^{th}-17^{th} centuries had already been introduced to tobacco through European trading trails, tobacco usage might be one of the reasons for a relatively low microbial diversity within historic dental calculus samples of postmedieval Latvians.

Another interesting finding Clemente et al. mentions is the presence of environmental bacterial taxa, such as *Knoellia* or *Solibacteriaceae* in the profiles of skin microbiota of Yanomami (Clemente et al., n.d.). These taxa, previously reported as environmental, appeared to rightfully occupy their niche within human skin microbiome, causing no harm and, most likely, providing some beneficial functions for community and for the host (Groth et al., 2002; Ward et al., 2009). Similar observation was made recently linking rise of colon cancer in western civilization to shortage of exposure to nature (Bolourian and Mojtahedi, 2018). *Streptomyces*, predominantly soil bacterial genus, appear to be healthy members of nonhumans, whose microbiomes might represent a snapshot of human microbiomes in past hunter-gatherer and farming environments. It is suggested, that *Streptomyces*, producing antiproliferatives/immunosuppressants, could protect our ancestors from inflammatory bowel diseases and subsequently lower their susceptibility to colon cancer (Bolourian and Mojtahedi, 2018).

In 2019 Winfried Blum et al. proposed a novel environmental microbiome hypothesis, stressing out a close linkage between soil microbiome and human intestinal microbiome (Blum et al., 2019). The hypothesis discusses co-evolution of the two microbiomes as well as the impact they have on one another. It would be fair to say, though, that the idea of soil-gut connection existed earlier and there is a clast of studies, focusing on this topic. David and Charles F Sing in 2010 suggested that soil exposure might provide important epigenetic signals, shaping our microbiome and our overall health (Sing and Sing, 2010). One of co-evolution arguments is believed to be well-documented practice of geophagy cultural tradition – soil dietary consumption, willful or accidental (Derbyshire, 2007; Johns and Duquette, 1991). Hypothesis also have been tested on animal models, suggesting that the diversity of gut microbiome increases being in contact with non-sterile soil, while contact with sterile soil

leaves host microbiome unaffected (Blum et al., 2019). Another recent study, performed in 2019 by Laura Grieneisen et al., explored gut microbiome of terrestrially living baboons. It was concluded that soil might be the most influential factor on animal gut microbiome, affecting its formation 15 times more strongly than the genetics of the host (Grieneisen et al., 2019).

A growing evidence also suggests that traditional farming practices with exposure to diverse healthy soils positively influences innate immune response and lowers the risk of developing allergies and autoimmune diseases (Stein et al., 2016). This intersects with yet another hypothesis of environment-human health correlation: Hygiene Hypothesis. First formulated in 2002, it suggested that contact with unhygienic conditions early in life may further protect individual from allergies by strengthening T helper 1 (TH1) cells (Yazdanbakhsh et al., 2002). Later, addition of microbiome perspective once again stated the contact with diverse environmental microorganisms to be beneficial to one's internal microbiome diversity and thus provide another immune system adaptation lever (Ege, 2017). Subsequently, it can be concluded that by contacting with microbe-rich environments like soil we nurture our adaptation abilities through different mechanisms at the same time. We enrich our microbiome, helping it regain healthy diversity and we introduce pathogens to our immune system which can further contribute to immune tolerance by stimulating immunoregulatory pathways (Wall et al., 2015).

Decades-long continuous loss of our ancestral microbial diversity together with important microbiome members due to urbanization, hygiene and antibiotics nowadays intersects with global pandemics control measures. Even more intensified hygiene, physical separation, travel barriers and self-isolation are expected to have substantial long-term effect on human health, preventing microbial diversity acquisition and accelerating diversity decline (Domingues et al., 2020; Finlay et al., 2021). Taking into consideration microbiome's influence on human health it is vital to track both how microbiome influences one's susceptibility to coronavirus and how pandemics preventive measures might affect global health in the long-term. This information might prove to be useful in disease prevention and treatment as well as dealing with long-term pandemics consequences (Finlay et al., 2021).

Since the invention of penicillin in the beginning of 20th century germ theory reigned over medicinal practices. One of the main paradigms of germ theory is treating the disease-causing agent while not defining the host. The idea behind it is that if you can find the disease-causing element, then you can decrypt what measures should be taken to eliminate it, this way curing the disease. The theory works very well helping us fight against infectious agents, however the decline of infectious diseases in 20th century clearly matches the rise of noncommunicable chronic diseases (Egger, 2012).

Modern realm of the rise of chronic diseases exposes the fact that the reigning medicinal paradigm might have some aspects that are subject to improving. Chronic diseases cannot be characterized by one shared etymology. It seems like to tackle this puzzle we need a paradigm shift, that would allow us to look at the problem from a different angle. In the context of chronic diseases and our continuously emerging notion about microbiome, this paradigm shift might redefine how we see human health: from disease as an invader to health as a process. To successfully implement this paradigm shift we might have to take few steps back and rethink our relationship with nature – it appears that our mind evolves faster than our body, which is still following some hunter-gatherer scripts, created many centuries ago. Within these scripts lies a holistic view of a human being – human body's systems are not discrete entities but one connected superorganism. It is much like one complex ecological system – if you disrupt one part of the system, soon every other part is affected. The idea of wholeness expands even further, beyond one individual human being, merging us with environment we live in: as we see, we share same problems. For example, as discussed above, human intestines share functional similarities with soil rhizosphere and microbiomes of both biotopes appear to have a functional linkage as well (Ramakrishnan et al., 2021).

Nonetheless, future studies and novel approaches are needed to test these ideas. Human microbiome is one of the most dynamic biomolecular research topics with wide areas for potential investigation and relevance in preventive medicine. Despite various methodological challenges, archaeological human remains specimens provide us the opportunity to access snapshots of ancestral microbial profiles and study microbial-host coevolution.

5. Conclusions

1. Human archaeological bone samples can serve as a secondary deposit material for human microbiome remains, trapping human microbial agents during the process of soft tissue decomposition and successfully storing them for centuries.

2. Archaeological dental calculus provides a reliable snapshot of historical human oral microbiome.

3. Bacterial species diversity of postmedieval Latvian dental calculus microbiome does not have a statistically reliant difference from modern Latvian dental calculus microbiome.

4. Oral biofilm type can have a greater impact on microbial communities than chronological origin of the sample.

5. Human dental calculus can store the remains of historic pathogenic bacteria.

6. Human teeth samples provide a relatively good average yield of endogenous human aDNA.

7. DNA fragment size selection strategy could not easily eliminate the impact of the possible presence of eDNA in ancient microbiome datasets.

8. Soil microbiome represents a challenging factor in ancient human microbiome research as no existing methodological technique is capable of distinguishing authentic human microbial remains from ancient environmental microbial remains.

6. Thesis

1. Archaeological human remains material captures and stores human related microbial DNA.

2. Historic dental calculus microbiome from postmedieval Latvian population is similar to modern Latvian dental calculus microbiome in terms of both alpha and beta diversity.

3. A fraction of environmental bacteria found within all sample groups could potentially represent members of historic human microbiome.

7. Publications

1. **Kazarina, A.**, Gerhards, G., Petersone-Gordina, E., Kimsis, J., Pole, I., Zole, E., Leonova, V., Ranka, R., 2019. Analysis of the bacterial communities in ancient human bones and burial soil samples: Tracing the impact of environmental bacteria. Journal of Archaeological Science 109, 104989. https://doi.org/10.1016/j.jas.2019.104989

2. **Kazarina, A.**, Petersone-Gordina, E., Kimsis, J., Kuzmicka, J., Zayakin, P., Griškjans, Ž., Gerhards, G., Ranka, R., 2021. The Postmedieval Latvian Oral Microbiome in the Context of Modern Dental Calculus and Modern Dental Plaque Microbial Profiles. Genes 12, 309. https://doi.org/10.3390/genes12020309

3. **Kazarina, A.**, Kimsis, J., Petersone-Gordina, E., Zayakin, P., Poksane, A., Gerhards, G., Ranka, R., 2021. Exploring DNA preservation and taxonomic diversity in postmedieval human tooth samples in Latvia. Journal of Archaeological Science: Reports 40, 103213. https://doi.org/10.1016/j.jasrep.2021.103213

Related publications:

4. Pētersone-Gordina, E., Gerhards, G., Roberts, C., Jakob, T., Ranka, R., Kimsis, J., Zole, E., **Kazarina, A.**, 2021. Differential diagnosis of abnormal enlargement and bending deformities in the skeleton of a medieval child from St Peter's Church cemetery, Riga, Latvia. Anthropologie. https://doi.org/10.26720/anthro.21.03.30.1

8. Approbation of research

1. **Kazarina A**, Gerhards G, Petersone-Gordina E, Kimsis J, Zole E, Leonova V, Zajakins P, Ranka R. "Ancient human oral microbiome data mining by using dental calculus of post-medieval archaeological remains from Latvia" 2019 The 10th International Conference of Prof. Jonas Puzinas. Investigating bones: diet, health, environment in the Baltic region. Vilnius, Lithuania, 2019

2. **Kazarina A** "Archaeological dental calculus as a record of ancient human pathogens" 2018 Latvian Biochemical society conference, Riga, Latvia, 2018

3. **Kazarina A**, Gerhards G, Petersone-Gordina E, Pole I, Leonova V, Igumnova V, Kimsis J, Capligina V, Ranka R. "Insights into archaeological human sample microbiome using 16S rRNA gene sequencing" 2017 IEEE International Conference on Bioinformatics and Biomedicine (BIBM), Kansas City, Missoury, USA, 2017

4. **Kazarina A**, Gerhards G, Petersone-Gordina E, Leonova V, Pole I, Capligina V, Jansone I, Ranka R, "Metagenomics analysis of archaeological human remains samples from Medieval Latvia as a primary screening tool for the identification of pathogen genomes", 2nd Conferene "Rapid Microbial NGS and Bioinformatics: Translation Into Practice", Hamburg, Germany, June 9-11, 2016

5. **Kazarina A**, Gerhards G, Petersone-Gordina E, Pole I, Zole E, Vilks K, Capligina V, Jansone I, Ranka R, "Next Generation Sequencing of Ancient DNA for the identification of the Mycobacterium tuberculosis genome in human remains", 40th FEBS Congress, July 4 – 9 2015, Berlin, Germany. Thesis published in The FEBS Journal 282 (suppl. 1) (2015) p 362.

6. **Kazarina A**, Japina K, Keišs O, Salmane I, Bandere D, Capligina V, Ranka R, "Tick-borne encephalitis virus prevalence thru I. ricinus ticks in birds in Latvia during autumn migration" Riga Stradins University (RSU) Scientific Conference, Riga, Latvia, 2014, Poster. Thesis published in RSU Scientific Conference Book of Abstracts, 2014, p182.

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