Putting dental calculus under the microscope

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Putting Dental Calculus Under the Microscope

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Jan Bartholdy

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Preface

This is not a traditional dissertation, which was a conscious choice on my part. First of all, it's not very common for a dissertation in my faculty to have a preface, which is why I have prefaced this preface with an explanation for why I need a preface. This mainly explains decisions regarding the format and style of my dissertation rather than the scientific content, which is why you won't see the phrase 'dental calculus' here. Oh, shoot...

Feel free to jump directly to Chapter 1 if you don't want to read this.

When I started my PhD research I had no intentions of shaking things up. I was going to put my head down and do my research, publish my articles in traditional journal venues, create a traditional article-based dissertation, and finish in the allotted four years. Six years later, and I accomplished... well, none of the above. Along the way I got a look behind the curtain of academic publishing. I didn't like what I saw. Not even a little bit. This was fueled by an introduction to Open Science. Science in the context of Open Science just made sense to me. This caused some delays as I dove head first into an Open Science rabbit hole. Also, covid. At first I vowed (to myself and those around me who would listen) never to publish any of my papers in Evilseer. Then, I took it a step further and vowed the same for more major publishers, including Springer and Wiley. Why do we pay publishers to take our copyright, publish our research, then pay extra so we're allowed read it? You may not be paying out of pocket, but your library is likely

covering those costs with expensive subscriptions. I'm sure they would much rather use that money on more useful stuff. All this to say, you won't find any of my PhD papers in the traditional journals. I wanted to try different platforms, like preprint servers and PCI_Archaeology.

Around the beginning of my PhD research I was also introduced to R statistical software. I can no longer remember how this came about, but after many months of rage-quitting and returning to SPSS, vowing never to open R again, I started to see the value of using scripting languages (and free, open-source software) for statistical analysis. It turns out when you have a document outlining every step you made in the analysis, it's easy to reproduce; both by yourself and others. Who knew? No need for the same 'point and click' all over again. I used R Markdown for most of my output, website, presentations, articles, etc. Then I took it a step further and started writing my dissertation in R Markdown (and eventually Quarto). My dissertation was now fully reproducible, and could be rendered in different formats with little change to the documents with the actual content. One of these formats was HTML. I could turn my dissertation into a website. That was pretty cool. I could have a dynamic, outward-facing dissertation easily modified when needed. This series of events led me to publishing my dissertation online, before it was completed, as a way to show the progress to the world. Of course most of the world didn't actually care, but a few people thought it was a pretty cool idea; and, more importantly, it made the writing part enjoyable. Or at least as enjoyable as something that's not very enjoyable in the first place. It definitely motivated me to make continuous progress. The (theoretically) wide availability of my dissertation made me start thinking about accessibility. This means increasing the readability and legibility of the dissertation, not only with the formatting, but with the language used. This doesn't necessarily mean that it can be easily picked up by someone with limited knowledge of the field. Writing 'academically' is not just exclusionary to members of the public, but also to those for whom English does not come naturally. Plus, I've found it to be a tedious read, even as a native English speaker. In my experience, writing more accessibly also requires a deeper understanding of the

subject matter.

Open Science is a priority in all of my work and will be reflected in this dissertation; sometimes directly, sometimes indirectly. Admittedly this is occasionally taken to an extreme: A fully reproducible dissertation, publishing everything before it's actually done, and avoiding traditional journals. Ultimately I was just fed up with the status quo. We as researchers need to do better. Contributing to knowledge requires more than having a paper accepted in a 'prestigious' journal. We need to ask ourselves why we are doing science, and for whom we are doing it.

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Acknowledgements

Where to begin? So many people helped shape this thesis, and therefore I do not take full responsibility for the quality (or lack thereof) of this work.

First of all, my understanding supervisor, Dr. Amanda Henry, who waited patiently through delays caused by covid and two kids. Not to mention supporting all my non-traditional ventures in the name of Open Science and accessibility. prof. dr. Annelou van Gijn for providing feedback on experiment design and dissertation drafts.

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Dr. Ben Marwick and Dr. Esther Plomp, whose passion and commitment to Open Science inspired me to make all of my work as open and transparent as possible. This also likely contributed to some of the delays; so, thanks for that.

My colleagues at TU Delft (Yasemin and the Data Steward team, especially) who were very encouraging about finishing my dissertation while working a part-time

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Anouk, Marie, Supriya, and Nina for having the patience to be friends with a PhD student with two small children. Femke and Maia for being great and motivating office mates.

Of course, I have to acknowledge my family for their moral support, and since they are the most likely to read this. Liam and Oliver, for making everything a bit more of a challenge.

Finally, my dad. An unlimited source of support and guidance through the whole process. I couldn't have done it without you. I only wish you could have been here to see me finish it.

Open Science Statement

All materials and data, including the source code for the dissertation itself, are made available to the best of my ability. All articles in association with the dissertation are/will be Open Access.

All outputs can be found, either directly or indirectly, on the Open Science Framework (DOI: 10.17605/OSF.IO/3YX8M).

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1 | Introduction

Dental calculus is becoming a popular substance in research on the behaviour and biology of people in the past. You may also know it as tartar or mineralised plaque. In other languages the word is often related to “tooth stones”. In fact, calculus is itself latin for ‘pebble’. This was originally used as a term for mathematical calculations using counting stones, and only later used to describe various calcifications in the human body (https://www.etymonline.com/word/calculus). This can be the cause of some confusion, as calculus is also a branch of mathematics. If you see the term ‘calculus’ in this dissertation, you can safely assume that I’m referring to stuff that grows on your teeth and for which you receive lectures from your dentist, and not the topic you dreaded in high school.

I will briefly describe the formation of dental calculus here, but for a more thorough review of the entire process I refer you to Chapter 2. Dental calculus is formed from dental plaque, a substance that grows on your teeth and consists mainly of bacteria and a surrounding structure called the extracellular matrix. When the local environment within and around the plaque reaches a favourable alkaline pH, both the extracellular matrix and bacteria within will calcify (Jin & Yip, 2002; D. J. White, 1997). The alkaline pH causes minerals (especially calcium and phosphate) from saliva to enter the plaque, causing the extracellular matrix and eventually also the bacteria to harden, resulting in a concrete-like deposit on the surface of the teeth. The process repeats itself when new bacte-

ria colonise the surface of the newly formed dental calculus, creating a layered structure, though somewhat disorganised (Akcalı & Lang, 2018; Jepsen et al., 2011). Dental plaque can accumulate more easily on teeth (and dental calculus) because they are a hard, non-shedding surface. Most of the surfaces in our mouth are covered by a layer of cells called the oral epithelium. These cells are continuously renewed as new cells are formed and dead cells fall off (Squier & Finkelstein, 1998). This constant turnover means that it is difficult for bacteria to build the communities they require for producing biofilms. Enamel, the white substance that covers the crown of your teeth, behaves differently. It stops growing when the tooth has fully formed. After that, there is no renewal. This allows bacteria to continue to grow and develop communities if there is no intervention from you (or your dentist). Dental plaque can trap a variety of different microparticles, including bacteria, human proteins, and small debris from the food we eat (De La Fuente et al., 2013; Hendy et al., 2018; Henry & Piperno, 2008). When the plaque mineralises, it can preserve these microparticles over long periods of time, even after the person whose teeth provided a home for the calculus has died. Also, the main crystal structures in calculus strongly bind DNA, making calculus a fantastic source of ancient DNA (aDNA) from the mouth (Warinner et al., 2015). Another advantage of dental calculus is that it represents a more recent and direct source of diet than teeth or other bones. While bones and teeth can take years to remodel and incorporate a dietary signal, calculus forms on a much smaller timescale and is in direct contact with the dietary material. Calculus can form within weeks at any point during an individual's life and may, therefore, indicate a recent and direct consumption of food, while bone can take years to show a (indirect) dietary signal, following food molecules entering the bloodstream, and finally entering the bone from there. Further, enamel stops forming after the crown of the last tooth has developed—third molars, or 'wisdom teeth—at around 16 years of age, and the turnover of dentin is very limited (Hillson, 1996). These properties are probably why archaeologists have become increasingly interested in dental calculus.

1.1 Dental calculus in archaeology

The main archaeological interest in dental calculus is to explore research questions involving diet and the evolution of the oral biome and oral health. To this end, it can contribute a surprising amount for such a small, seemingly insignificant material. This relates to its ability to retain and preserve a wide variety of different materials, from the food we eat to the bacteria that make their home in our mouths (Adler et al., 2013; Fellows Yates et al., 2021; Henry & Piperno, 2008; Warinner, Rodrigues, et al., 2014; Warinner, Hendy, et al., 2014). The goal of current studies targeting archaeological dental calculus have not changed much since the early uses of dental calculus in archaeological research, but the methods certainly have, allowing us to unearth information that was previously not considered possible. By my count, archaeological dental calculus has now been subject to various forms of microscopy (Charlier et al., 2010; Middleton & Rovner, 1994; Robert C. Power et al., 2022); extractions of biomolecules including DNA, proteins, and metabolites (Adler et al., 2013; Warinner, Hendy, et al., 2014); and stable isotope analyses.

Perhaps the most common use of dental calculus is to recreate the diet of past people and populations (Figure 1.1B). One of the ways to do this is by dissolving the calculus in a weak acid or decalcifant, or mechanically breaking it up. This process releases any fragments of plants that were trapped within the calculus and can be identified, for example with a microscope. The tricky part is not destroying the plant fragments when releasing them from the calculus. As far as I can tell, the first attempt at this was the extraction of phytoliths (silicified plant remains) from the teeth of cows, sheep, and horses (Armitage, 1975). This was a somewhat isolated use-case, and the method didn't really catch on until the 1990s (Ciochon et al., 1990; Middleton 1990, in Middleton & Rovner, 1994). The first extractions from human teeth followed shortly (Fox et al., 1996), and there are now studies using plant microremains (especially starch granules and phytoliths) from dental calculus to infer diet in past peoples from across the world, including Pacific Islands (Dudgeon & Tromp, 2014), China (Chen et al., 2021),

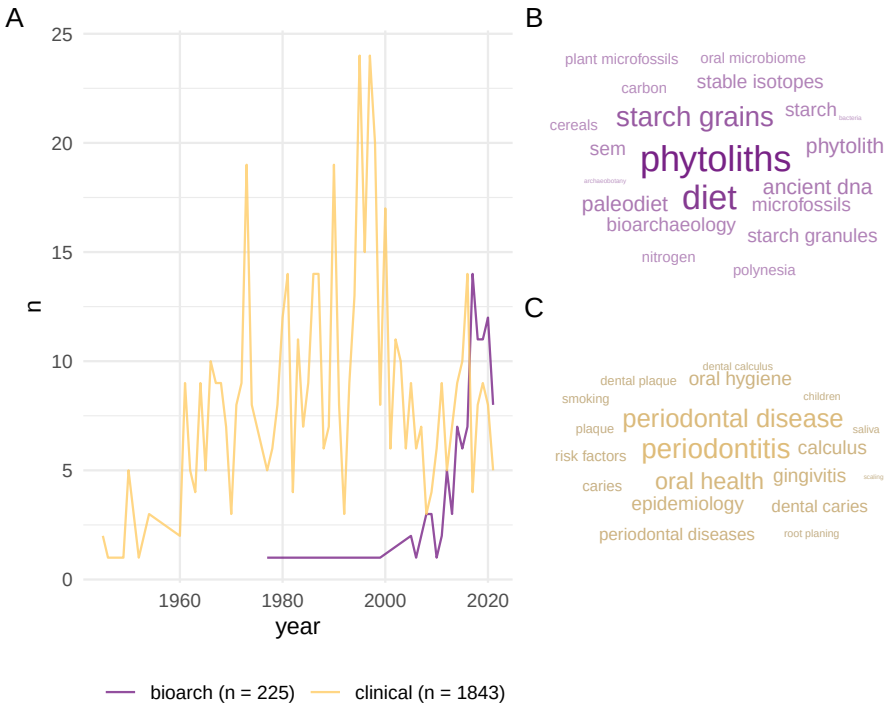

Figure 1.1 – Plot of the number of articles per year in bioarchaeology and clinical dentistry with the term 'dental calculus' in the title.

Europe (Fiorin et al., 2021), and more (Buckley et al., 2014; Henry & Piperno, 2008; Mickleburgh & Pagán-Jiménez, 2012). The durable nature of dental calculus also means that microremains within it can survive for millennia, allowing us to look at the diets of early humans and other hominins (Buckley et al., 2014; Chen et al., 2021; Hardy et al., 2009; Hardy et al., 2012; Henry et al., 2012, 2014; Henry & Piperno, 2008; Piperno & Dillehay, 2008).

That bacteria can become trapped within calculus has been known to archaeologists for a while (Brothwell, 1981, ; Vandermeersch et al., 1994), but it wasn't used in archaeological research until DNA extraction started to become more accessible (De La Fuente et al., 2013). Dental calculus then became part of the third scientific revolution in archaeology. The early studies focused on oral health in the past (Adler et al., 2013; De La Fuente et al., 2013; Warinner, Rodrigues, et al., 2014). Bacteria have shorter lifespans than humans which makes them useful when studying the evolution of bacteria in the human mouth (De La Fuente et al., 2013; Fellows Yates et al., 2021). Diet has also been a focus of paleogenetic research. This has mainly been addressed by considering how long-term changes in the patterns of bacteria within the mouths of our ancestors have changed that could be related to changes in diet. Just like we adapt to deal with various diseases, climates, etc., we also adapt to changes in our diet (Adler et al., 2013; Fellows Yates et al., 2021). Directly identifying genetic markers of plants and animals within dental calculus is difficult, but not impossible (see Warinner, Hendy, et al. (2014)). Most of the DNA within dental calculus will be oral bacteria, and this will overwhelm the small signal from plant DNA, which makes species identifications problematic (Fagernäs et al., 2022). A newer field of biomolecular archaeology, paleoproteomics, may be able to address this issue by targeting plant proteins, along with a range of other dietary protein sources. Hendy and coauthors were able to identify a number of these in dental calculus, as well as proteins from cereals, and milk proteins from different sources (Hendy et al., 2018). Dental calculus has also become a target for extracting other biomolecules that may be related to diet, such as alkaloids, fatty acids, and carbohydrates (Gismondi et al., 2020; Velsko et al., 2017). The

methods used for this have also proven to be useful in detecting compounds that are related to other activities and ceremonies, such as nicotine (Eerkens et al., 2018), and may provide some evidence of medicinal practices (Gismondi et al., 2020).

To a lesser extent, the presence and amount of dental calculus on teeth has been used as an indicator of dental health (Drewett, 1975; Lieverse et al., 2007; Sagne & Olsson, 1977; Zhang, 1982). Pilloud & Fancher (2019) explored the terms associated with a number publications on dental or oral health, dental calculus came up as one of them; albeit not the most common, which was (unsurprisingly) dental caries (Figure 1.2).

To a lesser, lesser extent, it has also provided some interesting insights on non-dietary activities, such as occupations and smoking habits. In a rare find, blue particles were detected in the dental calculus of a Medieval German woman. These blue particles originated from lapis lazuli, an exotic stone often ground into pigments and used to illuminate manuscripts (Radini et al., 2019). Nicotine was detected in dental calculus of pre-colonisation individuals from California using Ultra-Performance Liquid Chromatography Mass Spectrometry (UPLC-MS), showing direct consumption of tobacco and providing more detailed insights on the demographics of consumption in a way that no other human-adjacent archaeological materials can.

It wasn't always appreciated for the wealth of information hidden within its hardened shell. Until roughly 20 years ago, archaeologists who encountered calculus had limited use for this material. Some researchers quantified it using a simple four-stage scoring method that was developed for recording deposits on archaeological dental calculus (Brothwell, 1981), similar to a common clinical scoring system (J. G. Greene & Vermillion, 1964). The four-stage system is probably still the most widely used among archaeologists. More detailed methods are also available (Dobney & Brothwell, 1987; T. R. Greene et al., 2005), but the original method is generally preferred for its simplicity. Unfortunately, knowing the size of a calculus deposit is not as valuable as being able to analyse the deposit

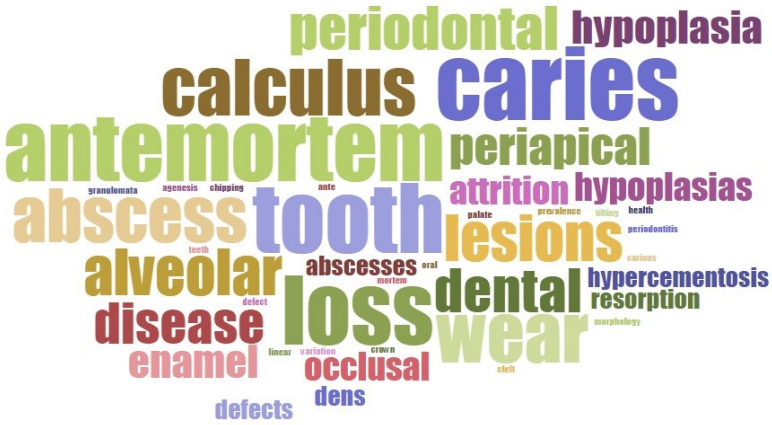

Figure 1.2 – Word cloud of most common dental terms in articles. Figure is from Pilloud & Fancher (2019), Figure 1.

itself, and the deposits were often removed because they obscured tooth and root morphology (Scott, 2015). This had made a lot of people very angry and been widely regarded as a bad move (Adams, 2002, p. 1). Hindsight being what it is, it's hard to blame anyone. A lot of dental research mainly focuses on the prevention and removal of dental calculus.

The wide range of applications for dental calculus that we know about today, and the fact that it's pretty much ubiquitous in the past thanks to poor oral hygiene, makes it a really exciting target for future (and current) paleodietary research. That being said, the study of dental calculus doesn't seem to fit into any predefined areas of study within (and beyond) archaeology. Most researchers seem to see it as a means to the information contained within, rather than being worth studying in its own right. This can be problematic. Other than what we can see with our current methods, what do we really know about dental calculus and how its growth and structure affect the reliability of these methods and potentially distort our interpretations of the past?

1.2 What is dental calculus?

To answer these questions, we must first answer a single, surprisingly difficult question: What is dental calculus? I'm not referring to its formation or composition, which I briefly described above. How do we categorise it? Is it a dental disease? An oral health condition? A byproduct of oral conditions? We start by exploring various definitions of oral health. Definitions in an introduction are a little cliché and tedious, but often necessary. Since oral health is a complex topic, definitions of oral health are often purposefully (and confusingly) broad, and they extend beyond physical well-being and into the realms of emotional and social comfort. The World Dental Federation (FDI) defines oral health as the ability to perform mouth- and face-related functions with confidence and without pain (including smiling, speaking, eating, etc.) ("FDI's Definition of Oral Health | FDI," n.d.) (https://www.fdiworlddental.org/fdis-definition-oral-health). Both the

World Health Organisation (WHO) and FDI take a similar approach to defining oral conditions, giving a list of conditions that cause discomfort, pain, disfigurement, or death. The list includes the dental conditions tooth decay (caries), gum disease (periodontal disease), and dental trauma, but not dental calculus ("Oral Health," n.d.) (https://www.who.int/news-room/fact-sheets/detail/oral-health). While these are not likely to cause death, they are often the source of physical and emotional discomfort, and may cause further health complications if they are not dealt with in a timely fashion.

Dental calculus and dental plaque are not considered oral conditions according to WHO. In fact, dental plaque is part of the normal functioning of our oral biome (Marsh, 2006). When plaque reaches a certain level of acidity over a prolonged period of time, the normal functioning of the bacteria within the plaque may shift towards a disease-causing function. The biofilm will cause the surface of the enamel to demineralise, eventually resulting in a cavity (or caries). Dental caries are unequivocally considered a dental disease. If, instead, the biofilm calcifies, dental calculus is the result. Its status in oral health is questionable.

Dental calculus is not known to be painful, nor does it affect the ability to perform the functions listed above. However, with continued accumulation, it may affect the confidence of the person performing these tasks (Collins & Freeman, 2007), and in extreme cases it can affect function (Balaji et al., 2019). Most of the virulence and disease-causing potential is lost when the bacteria within dental plaque calcify (Akcalı & Lang, 2018). It has been shown to contain pockets of living bacteria that can be detrimental to oral and dental health (Tan, Gillam, et al., 2004; Tan, Mordan, et al., 2004). The rough, porous surface of dental calculus is also a great place for bacteria to attach more easily and develop a new layer of plaque on the surface of the calculus. This is likely why there is often a correlation (NOT causation) between dental calculus and periodontitis, especially subgingival calculus (Jepsen et al., 2011; D. J. White, 1997). Since it seems to fulfill some of the criteria of an oral condition, it should be considered as such, at least under the definitions provided by WHO and FDI. Whether or not

dental calculus can be considered an oral disease is more questionable. While it does grow on the surface of teeth, it doesn't seem to affect the underlying enamel. And while there is a relationship with periodontal disease (which has been defined as a dental disease), the nature of this relationship is still under debate, with calculus likely being a secondary contributor (Jepsen et al., 2011). As such, we can probably limit the definition to an oral condition and not necessarily a dental disease (Pilloud & Fancher, 2019). In fact, dental calculus is quite hard, so a layer of dental calculus on a tooth can actually protect it from wearing down (although there are better options).

1.3 The study of dental calculus

It seems that the researchers who are studying dental calculus approach it from a wide range of different fields and backgrounds, including genetics, proteomics, botany, and (bio)archaeology. The paleogeneticists mine it for the wealth of information it contains on oral health and disease in the past (Fellows Yates et al., 2021; Warinner, Rodrigues, et al., 2014). Paleodiet researchers extract microremains and residues from food (Henry & Piperno, 2008; Mickleburgh & Pagán-Jiménez, 2012) to infer dietary practices. Bioarchaeologists use its presence and amount to broadly infer diet, and dental and overall health in a given population (Belcastro et al., 2007; Lieverse et al., 2007; Novak, 2015; Šlaus et al., 2011; Yaussy & DeWitte, 2019). This leaves research output from studies of calculus scattered across multiple venues, with no clear gathering point. I think it's fair to say that dental calculus should be included in discussions of pathological oral conditions, even if its role is secondary. But who is currently studying dental calculus as a substance in its own right? And why do we need to learn more about it if we're just interested in what's inside? Related discussions have started to take place in recent years (Bucchi et al., 2019; Radini & Nikita, 2022; Wright et al., 2021).

The lack of a specific field of study for dental calculus to belong may be re-

lated to how it's taught to students (and if it's taught at all). Textbooks from the more established fields in bioarchaeology are probably a good indicator of the teaching curricula, which also impacts research focus. The most popular osteoarchaeology textbooks only briefly mention dental calculus as more of a footnote than anything else. A couple of lines describing what it is (usually 'mineralised plaque') and that it can contain food debris and bacteria T. D. White et al. (2011). They're not wrong. Diseases that manifest themselves in the skeleton as lesions on the bones have a very clear home in paleopathology. No one questions whether or not the degeneration of vertebrae from tuberculosis should be included in the paleopathology textbooks (at least not as far as I'm aware).

These textbooks often include chapters on dental disease, where more detailed descriptions of dental calculus are usually found (e.g. Roberts & Manchester, 2007; Waldron, 2020). Dental caries, calculus' more famous sibling, will often get a few pages. In some cases, dental calculus may even be hidden within a section on periodontal disease or plaque (Aufderheide et al., 1998; e.g. Ortner, 2003). The focus of these (sub)sections is varied, with some simply describing what it is, and others giving brief discussion on the relationship between calculus and periodontal disease. A more detailed section was dedicated to dental calculus in *Ortner's Identification of Pathological Conditions in Human Skeletal Remains*, with a detailed description of formation, structure, and application in (biomolecular) archaeology (Kinaston et al., 2019). The description extends well beyond any (paleo)pathological significance of dental calculus. Can we fault the authors/editors for not giving it more attention? After all, it's not a dental disease, and its relationship with other dental diseases is unclear. What is clear, is that it has implications for oral health, and, for that very reason, could be addressed more extensively in paleopathology; certainly in the textbooks that include dental disease.

On the surface, dental anthropology seems like a more suitable home for the study of dental calculus. However, it's not included in *A Companion to Dental Anthropology*, an otherwise great resource on studying archaeological teeth.

The editors briefly acknowledge the valuable information gained from calculus and that it holds a lot of potential; but that's it (Scott, 2015). Other notable absences include textbooks such as *Technique and Application in Dental Anthropology* and *New Direction in Dental Anthropology* (Townsend et al., 2012), both of which dedicate considerable attention to dental caries. Hillson's *Dental Anthropology*, a book that I consider to be the 'bible' for dental anthropology, has a section on dental calculus in the Dental Disease chapter. It covers a basic description, the composition, microscopic structure, methods used for recording archaeological calculus, and the distribution in the dentition (i.e. which teeth are more prone to calculus buildup) (Hillson, 1996). Considering these are entire books devoted to the dentition, it seems odd that there is often only a few paragraphs (if that) on dental calculus. Granted, the only function teeth serve in the growth of dental calculus is as a suitable surface on which to attach; though the role of substratum is an important role, as dental calculus is seemingly unable to form on other surfaces in the oral cavity.

Since the use of dental calculus in biomolecular archaeology is relatively new, there are fewer available textbooks, and it rarely has a dedicated course. The most common place to find descriptions of dental calculus is, therefore, journal articles. There will be a short paragraph on dental calculus formation (and sometimes composition) in the introduction section. These are quite variable and are often limited by the word count of the journal. Despite this, the descriptions will often be as long, if not longer, than the sections in textbooks devoted to dental calculus (Velsko et al., 2019). The focus of these paragraphs are generally the same. They describe the formation and mineral composition of dental calculus, and provide some examples of how dental calculus has been used in related studies (not unlike the beginning of this chapter). The contribution of dental calculus to archaeology has been significant, so it is likely to receive more and more attention going forward. In fact, an entire chapter was recently devoted to dental calculus in the second edition of *Handbook of Archaeological Sciences* (Fagernäs & Warinner, 2023). Take that, dental caries!

1.4 The challenges of studying dental calculus

What we know about dental calculus and the influence of diet was reviewed in an article aimed at (bio)archaeologists. The overall conclusion reached in the article: it's still pretty unclear (Lieverse, 1999). Now, 20-some years later, there has been limited progress on this point. High-protein diets are linked to an increase of urea, which is linked to an increase in oral pH, which is linked to mineral deposition (Dibdin & Dawes, 1998; Wong et al., 2002). BUT, protein may also inhibit crystalisation (S. Hidaka & Oishi, 2007). Starch consumption has been linked to increased rates of caries in early farming populations (Storey, 1986). This is consistent with *in vitro* testing, at least for starches high in amylose content. So a high-starch diet causes caries, not calculus, right? Well, starches with a high amylopectin content are linked to increased calcification (S. Hidaka & Oishi, 2007). It likely depends on what is consumed along with the starch (Saburo Hidaka et al., 2008). There is also some (*in vitro*) evidence to suggest that silica may promote dental calculus formation by promoting mineral precipitation, i.e. the transfer of minerals from saliva to the biofilm (Damen & Ten Cate, 1989). Overall, this is an understudied area in both clinical and archaeological contexts.

Another aspect of diet and dental calculus where we are still looking for answers, is the process that causes fragments of food and other environmental materials to become entrapped in the dental calculus. We know that it happens. Decades of research has shown dental calculus to be a seemingly unlimited resource for dietary substances. We don't know exactly how this happens, and herein lies the potential for bias. Efforts have been made to understand how much of the consumed food makes it into the calculus. These include studies on modern humans (Leonard et al., 2015) and non-human primates (R. C. Power et al., 2015; Robert C. Power et al., 2021), where food intake is meticulously documented, and calculus subsequently analysed. These studies have common findings; the amount of the diet that becomes trapped in the dental calculus of any one person has no clear relationship to the amount of food that was

consumed. The most likely reason is that the formation of dental calculus differs between people (R. C. Power et al., 2015). So, it's not a great way to study the diet of a single person, but generally suitable to study patterns in the diet of a population. The more people you study, the more likely you are to gain a complete picture of the diet in a population. The fact that we can still see (in some cases, literally) remains that were consumed thousands of years ago is pretty cool. We just need a better understanding of why the record of diet from dental calculus differs from the actual intake of food. This will allow us to make more robust interpretations about past dietary practices. Something that may influence the dietary record that we get from calculus is the method we use to extract the dietary remains from calculus. Our understanding of dental calculus extraction methods is improving, with studies looking at the effect of various acids used to dissolve calculus (commonly EDTA or HCl) (Bucchi et al., 2019; Palmer et al., 2021; Soto et al., 2019; Tromp et al., 2017); as is our understanding of how the choice of tooth may affect our results (Fagernäs et al., 2021), and that not all we see is related to deliberate consumption (Delaney et al., 2023).

These studies provide valuable insights into potential biases of our sampling methods and the representation of diet within dental calculus, with a minor caveat. Most of these studies have been conducted on living primates or archaeological remains. An issue with using living (or once living) organisms is the inability to control factors related to the variability between subjects. Basically, studying humans is messy and complicated because we're all unique. It's a lovely sentiment but it can make for some messy science. Not bad science (not at all!). Just messy. A method of study that offers more control is the growth of plaque and calculus in a lab. This allows us to control many of the things that are difficult to control in humans, such as the bacteria that colonise our mouth, where each person has a pretty unique makeup of bacteria. We also have a very unique genome (with the exception of identical twins) that plays a role in how quickly we form calculus in our mouth (if at all). Certain enzymes start digesting our food as soon as it enters our mouth, and the activity of these enzymes fluctuates throughout the day, causing a lot of variability both within and between

individuals. Finally, the number of microremains that enter our mouth over days, weeks, and months, can be very different between people, even with the same diet. All these things can muddy the results of research on living subjects, where a lab-grown approach can help tease out confounding factors. I don't believe research conducted on lab-grown biofilms can in any way replace studies with modern or archaeological individuals, nor should they. But it can complement these studies by zooming in on certain aspects that are too difficult to isolate in (once-)living people.

Often we can draw from clinical studies as there are common goals, e.g. discovering the aetiology and/or presentation of a disease. However, the motivation driving the studies in archaeology and dental research are inherently different; although, there is certainly overlap in some areas (Figure 1.1B and C). There is more interest in preventing dental calculus from forming in the first place, so most studies focus on short-duration models to explore anti-microbial treatments and inhibition of biofilm formation and plaque buildup (Exterkate et al., 2010). As shown in a previous study, calculus and plaque have distinct microbial profiles (Velsko et al., 2019), so the applicability of short-term models to explore archaeological questions on dental calculus are limited, since plaque is rarely (if ever) preserved. Archaeologists are more interested in questions related to how diet influences the growth of biofilms, and how fragments become embedded inside, and what we can say about diet. Further, the interest in dental calculus as a field of clinical research has been declining since the 2000s, which, as far as I'm aware, is when the last studies growing dental calculus in a lab were conducted. We can see this by the number of clinical articles with the term dental calculus in the title (Figure 1.1A). And they certainly aren't interested in how food debris becomes trapped inside our calculus. Dental calculus has also become less of a problem with the use of modern dental hygiene practices and regular visits to the dentist (Velsko et al., 2019).

To summarise: Bioarchaeologists are interested in how dental calculus relates to dental and general health; paleodietary researchers are interested in the food

remains that are trapped inside; paleogeneticists are interested in accessing the oral bacteria that have been fossilised within; clinial dentistry views it as a nuisance to be removed and, ideally, prevented from forming in the first place. This lack of systematic research specifically devoted to dental calculus as a substance, rather than a means to an end, leaves a lot of questions regarding the expected behaviour of dental calculus and how information from the past becomes trapped inside. To summarise the summary: we need to ask more basic questions about dental calculus.

1.5 Aims

This dissertation is a contribution to a dental-calculus-centric body of knowledge, and addresses a gap in the fundamental research on dental calculus to further our understanding of how we can use dental calculus to reconstruct the diets of people in the past. The main aim is the development, validation, and application of a calcifying oral biofilm model to improve interpretations on archaeological dental calculus. By developing a model system we can isolate the effects of confounding factors in dental calculus and diet, and explore new uses for dental calculus in paleodietary reconstructions through fundamental experimentation. I also aim to assess the potential and limitations of dental calculus to explore dietary activities of past populations.

Every decision we make, from sampling to statistical analysis, leads us down a unique path towards a different interpretation from the other possible paths in the multiverse of analyses. It's important we fully understand the path we take, to ensure that it is the right path given the limitations, and one that maximises the validity and detail of our interpretations.

With these aims, I hope to address the following research questions:

How can we improve the resolution of our interpretations using dental calculus on individuals and populations? We are stuck in the identification of compounds, and unable to speak to the quantity, since we know that it's not very

representative of a single individual.

Can we trust the system? (i.e., using dental calculus to reconstruct diet) Since we don't know the mechanism of incorporation, there are likely hidden biases and limitations of our methods as a result. We don't know the starting point, i.e., exactly what and how much was originally trapped inside, so we have difficulty validating our methods.

How can a model improve our understanding of dietary reconstructions using dental calculus? How can it address current challenges in paleodietary reconstructions, and can it help us produce a better understanding of how dietary intake relates to the record of diet we extract from archaeological dental calculus?

1.6 Thesis outline and structure

If you have made it to this point, you have probably read most of **Chapter 1**, in which I provide some context to the study of dental calculus in archaeology and identify some areas that could benefit from further investigation. **Chapter 2** provides some background information on oral biofilms and oral biofilm models in more detail than I can do in the research articles included in Chapters 3 and 4. So if you're already well-versed in oral microbiology, feel free to skip to Chapter 3. If not, I recommend picking up a textbook written by actual experts in the field of oral microbiology. If, for some reason, you can't access one of these, feel free to read **Chapter 2**. I suppose there are worse options than something written by a PhD student in archaeology. The chapter reflects the current knowledge of biofilms and the oral microbiome (as best I could summarise) at the time of writing, and no warranty is given for the inevitable new developments that will change what we now believe to be true.

To address the aims of the dissertation outlined above, I developed a protocol to grow dental calculus in a lab on plastic tubes instead of looking at the real stuff you normally find inside your mouth. The reason for using lab-grown biofilms

instead of humans is that the *in vitro* lab model offers more control over all the factors that go into the growth of dental calculus, at least in theory. The real world is messy, and sometimes you need to remove things from the real world to break it down and really get into the nitty gritty of how it works. There are many different kinds of biofilm models, including single species of bacteria, select species determined by the researchers (defined consortium), and multiple species from some natural source (the human mouth, for example). I will cover the different types of models in more detail in **Chapter 2**. Since there are many biofilm models to choose from, developing a new protocol may seem counterproductive; however, few are developed for long-term growth and even fewer with the purpose of mineralising the biofilm to form dental calculus. One of the exceptions involves a highly complex setup that is unlikely to be supported by budgets and facilities available to most archaeological laboratories (Sissons et al., 1991).

After developing a working protocol, the next step was to determine if the stuff I grew in the lab is actually dental calculus. Or at least something close enough that we can use it to explore our research questions. To do this, we (myself and coauthors) determined the mineral and bacterial composition of our model using Fourier Transform Infrared (FTIR) spectroscopy and metagenomic classification **Chapter 3**. We then compared the results of these analyses to naturally grown dental calculus, both modern and archaeological.

Being confident that our model looks and behaves like human dental calculus, we then set out to test some very basic behaviours of starch grains within dental calculus. **Chapter 4** is a research article where we 'fed' the biofilm with a known quantity of starch granules during the growth period to see if the input quantity/ratio matched the extracted quantity (or output). Those who are familiar with dental calculus research will not be surprised that it did not. The more interesting outcome of the study is the more detailed explanation of how the input and output starch quantities were mismatched.

Chapter 5 is a separate article, in the sense that it doesn't involve the biofilm

model in any way. Rather, it addresses the theme of the overall utility of dental calculus in archaeological research. We look at possible medicinal compounds in the dental calculus of a Post-medieval Dutch population. We employed Ultra High Performance Liquid Chromatography coupled with tandem Mass Spectrometry (UHPLC-MS/MS) to identify various compounds in dental calculus, including alkaloids and other compounds. It shows the potential of dental calculus to inform about past practices, but also highlights some of the limitations we are currently experiencing in the field. **Chapter 6** is a discussion on the limitations and future potential of dental calculus in the field of archaeology, and what biofilm models can contribute to our understanding of past diet.

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2 | Background

The human mouth, or oral cavity, contains many different types of surfaces on which bacteria can attach and grow. These surfaces are both hard (teeth) and soft (mucosa, tongue, gingiva), and are exposed to the external environment. For this reason, the conditions within the oral cavity can vary considerably, resulting in a unique range of habitats for a wide variety of microbes. In fact, the oral biome contains bacteria from over 700 different species, some of which still haven't been named, or even cultured. There are so many bacteria in our mouth that it's actually hard to determine how many there are at any given time, but most estimates are in the billions. Some like stable temperatures and lots of oxygen. Others are better at dealing with fluctuations in temperature and oxygen availability. Some can fend for themselves and take what they need from the environment. Others depend on the presence of other species to break down their food into smaller pieces. Some like acidity. Others like alkalinity. So how can they all seemingly thrive in the same place at the same time? The answer is biofilms.

As an archaeologist, you may be wondering why you need to know all this stuff. Dental calculus is the result of a very complex series of events that involves the physiology of saliva, particular diets, age, genetics, and a bunch of other things. To better understand what we see when we analyse archaeological dental calculus to get at diet, we need to understand all of the processes that went into

forming it in the first place. Only then can we begin to fully unlock its potential in reconstructing past diets. In any case, we all have mouths, so on some level I'm sure this knowledge will be relevant.

2.1 Oral biofilms

The concept of biofilms represents a recent paradigm shift in microbiology (Costerton et al., 1987, 1995). Previously, researchers believed that you could isolate the organism of interest and learn about its growth, metabolism, etc. They assumed bacteria would behave the same as a free-floating organism in a lab test tube as it would in a real-world environment (such as the human mouth). More recently researchers have discovered that the behaviour of bacteria differs when they are part of a larger community, compared to when they are grown in isolation. Biofilms consist of large, intricate, multi-species communities of bacteria enclosed in an extracellular matrix of their own creation. The ability to produce this matrix gives the bacteria living within it an adaptive advantage compared to free-floating (planktonic) organisms. It equips them with resistance to both antimicrobials (such as antibiotic medication) and immune responses from the host that would normally be detrimental to their ability to survive (Marsh, 2005; Marsh & Bradshaw, 1997). Resistance to varying conditions is especially important in the oral cavity, which is a site of frequent fluctuations in temperature, pH, and oxygen availability. The viscoelastic nature of the biofilm provides some protection against mechanical destruction and dislodgement caused by, for example, the tongue and dental hygiene practices (Peterson et al., 2015). It also allows them to acquire nutrients from outside the biofilm, as well as generate and distribute nutrients within the biofilm to the various communities of bacteria residing inside (Flemming et al., 2016). Biofilms are quite persistent structures, and very few surfaces exist that can completely prevent bacterial colonisation and biofilm formation (Renner & Weibel, 2011).

2.1.1 Dental plaque

Dental calculus forms from a specific oral biofilm known as dental plaque. After we clean our teeth, our saliva coats the surface of our teeth (enamel) with a layer of proteins known as the dental pellicle (or acquired enamel pellicle). The pellicle is a film that protects our teeth from both mechanical wear and chemical decay, but in doing so, provides a viable surface for microorganisms to attach and initiate biofilm growth (Yao et al., 2003). Biofilm formation goes through several, often arbitrarily defined, stages of growth. They are arbitrary because they are defined by the researchers who study them, but are also necessary as a foundation to explain the development of a biofilm. Rather than thinking about the stages as occurring sequentially, you should think of them as occurring concurrently across different areas of the tooth surface. Biofilm formation is a very dynamic process, and is often over-simplified in visualisations (not unlike Figure 2.1).

The pellicle contains molecules (known as adhesins) that enable specific bacteria to attach to complementary receptors on the pellicle, in a process called adsorption, not to be confused with absorption. The difference being that it simply attaches to the surface of the tooth rather than being sucked into the tooth. When the pellicle adheres to the tooth, it becomes a surface for bacterial attachment (Yao et al., 2003). The first bacteria to attach are known as early coloniser bacteria (or pioneer colonisers) and include *Streptococcus* species (spp.), *Actinomyces* spp., and *Haemophilus* spp. (Uzel et al., 2011; Zijnge et al., 2010). The initial attachment occurs when the random movement of bacteria and the flow of saliva brings them close enough to the pellicle to attach. Some bacteria have a limited, often random, ability to move if they have long tail-like structures known as flagella, but most are brought to the surface by saliva.

As bacteria approach the pellicle-coated surface of a tooth, there are both attractive and repulsive forces at work. Repulsion because both the bacteria and pellicle proteins have a net negative charge (Song et al., 2015), causing electrostatic repulsive force; and attraction from van der Waals forces. Bacteria may

”How many roads must a man walk down?”

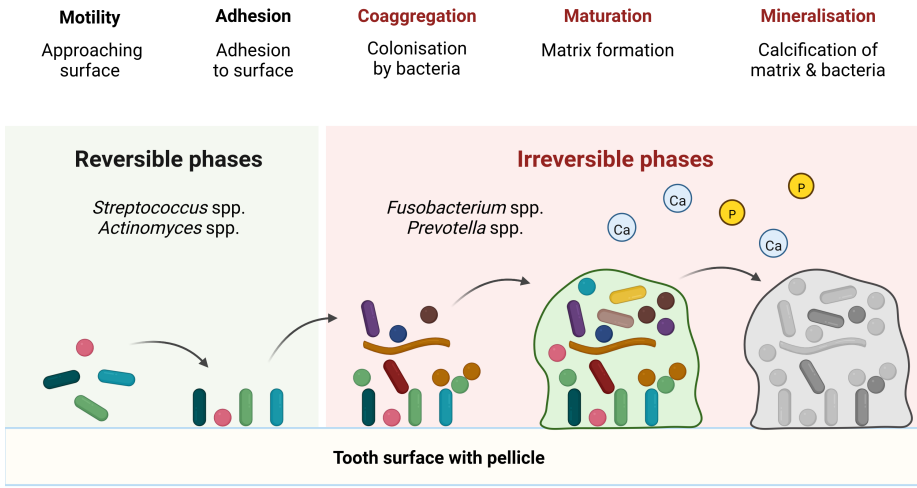

Figure 2.1 – A simplified overview of biofilm formation stages. Created with BioRender.com.

be more or less likely to attach depending on the distance from the bacteria to the surface. If the bacteria come too close to the surface, the initial attraction (primary maximum) will most likely be overcome by repulsion (primary maximum). Bacteria are more likely to attach when they encounter attractive forces at a further distance (secondary minimum), ultimately leading to a game of 'will-they-won't-they' between the bacteria and pellicle. This initial attachment is a weak physicochemical long-distance (10–20 nm; it's a long distance for bacteria) attraction; therefore, attachment is initially reversible, as bacteria can become detached by salivary flow or shearing action by the tongue (Marsh et al., 2016). This model of bacterial attachment, also known as the DLVO theory, can partially explain the aspects involved in microbial adhesion. Further explanation includes hydrodynamic forces, where hydrophobic components of the pellicle and cell surface interact (Bos, 1999; Vigeant et al., 2002). Overcoming the repulsive forces may be in part facilitated by motility in some organisms. The aforementioned flagellum, for example, may give the necessary 'push' to reach a region of net attractive forces (Jin & Yip, 2002). Additionally, the ionic strength of saliva may play a role in reducing electrostatic repulsion with increasing ionic strength (Renner & Weibel, 2011).

Attachment becomes stronger and colonisation becomes more solidified at a shorter distance, as surface molecules on the bacteria interact with complementary receptors on the pellicle, and the interactions between bacteria and pellicle become more direct. Some bacteria have components on their surface that allow them to attach directly to complementary components on the dental pellicle (adhesin-receptor interactions). These attachments are very specific because only certain bacteria have the right molecules on their surface (Jin & Yip, 2002). These receptors are often carbohydrates formed by the host, meaning us. Early colonisers are also able to attach to proteins and enzymes present in saliva, as well as onto the surface of other bacteria already attached to the pellicle (Jin & Yip, 2002; Nikitkova et al., 2013). When bacteria come within a shorter distance of the pellicle they may also attach directly to the surface with other hair-like structures (fimbriae) that are present on the surface of some bacteria. These

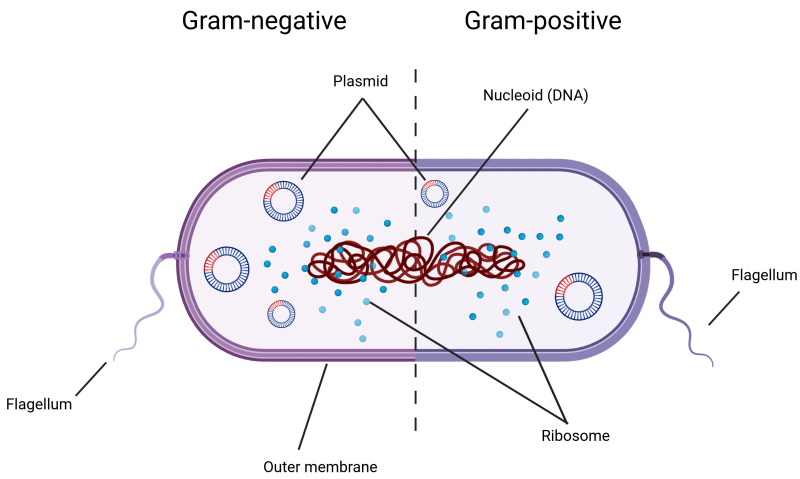

Figure 2.2 – General structure of a bacterial cell. Common features of gram-negative bacteria on the left, and common features of gram-positive bacteria on the right. Created with BioRender.com.

hair-like structures attach to matching receptors that are present in the pellicle (Nobbs et al., 2009).

While some bacteria specialise in attaching to surfaces, not all of them possess this ability. However, once the specialists have attached, they facilitate the adhesion of other bacteria (secondary colonisers) by allowing them to attach to their surface (coadhesion) rather than directly to the pellicle. For example, *Streptococcus gordonii* can attach to the pellicle and facilitate coadhesion with *Actinomyces naeslundii* (Palmer et al., 2003). Not all attachments involve proteins. They can also involve carbohydrates, enzymes, and various appendages on the surface of the bacteria, although these appendages often consist of proteins in their structure, for example the already mentioned pili and fimbriae (Nobbs et al., 2009). This can occur on a large scale, causing the number and types of bacteria on the tooth surface to grow, due to the ability of different species to attach to one another (coaggregation) (Jin & Yip, 2002; Marsh, 2006). Coaggregation and coadhesion are important parts of the growing oral biofilm. Most taxa don't have the necessary morphology to attach directly to a substrate, however most oral taxa CAN coaggregate with other species through cell-cell interactions, usually involving polysaccharides on the bacterial-cell surfaces (Kolenbrander et al., 2010; Palmer et al., 2017).

As the biofilm formed by early colonisers grows through continued multiplication and coadhesion/coaggregation, the diversity of the biofilm increases. The proportion of early-colonising streptococci gradually decreases while there is an increase of *Tannerella forsythia*, *Actinomyces* spp., and *Fusobacterium nucleatum* (Zijnge et al., 2010). *F. nucleatum* is a bacterium also known as the 'bridging species', as it's believed to play an important part in linking together early and late coloniser species—including *Prevotella* spp., *S. gordonii*, and *Porphyromonas gingivalis*— which might not otherwise be able to coaggregate (Kolenbrander et al., 2010; Kolenbrander & London, 1993). The increasing diversity of bacteria adhering to a surface results in communities of bacteria with the ability to communicate with each other, distribute nutrients, and alter the lo-

cal environment for more favourable conditions. This is made possible by the presence of an extracellular matrix, formed by the production of polymers by certain bacterial species (Marsh, 2010). Microenvironmental changes can allow species to survive in otherwise unfavourable environments; for example, the survival of many obligate anaerobes in an environment which is largely aerobic (oxygen continuously enters the oral cavity as we breathe). Bacteria with the ability to consume oxygen and produce carbon dioxide allow bacteria with a lower oxygen tolerance to thrive (Marsh, 2005). In fact, dental plaque predominantly consists of obligate and facultative anaerobes and is especially true for periodontitis-associated biofilms, which tend to be dominated by more species with a lower oxygen tolerance than their non-periodontitis counterparts (Curtis et al., 2020). A pH balance may be maintained by species that are able to consume acidic metabolic products produced by other species, and convert them to weaker acids. *Veillonella* spp. especially (Marsh, 2005). Metabolic products of some bacteria are used by others as nutrients. By-products of urea metabolism can be used by some organisms, who further break down the by-products, which can be used by yet other organisms (Flemming et al., 2016). Working as a community can increase survivability in the harsh and dynamic environment of the oral cavity, with rapid changes in pH, oxygen, nutrient availability, etc; though, extended fluctuations in environmental conditions can alter the composition of biofilms (Huang et al., 2012, 2017).

Perhaps ironically, an important part of the maturation of a biofilm is the removal of bacteria from the biofilm itself. Removal can occur through both internal and external mechanisms. It's likely that there is a continuous loss of microbes near/on the surface of the biofilm caused by shear forces from saliva and mechanical removal by the tongue. There can be multiple motivating factors involved in the active detachment by bacteria, including increasingly adverse conditions within the biofilm, such as nutrient depletion or an unfavourable local environment. If sufficiently adverse conditions persist, certain bacteria may make the active decision to 'peace out'. Dispersion of bacteria from a biofilm requires production of matrix-degrading enzymes, and, as such, not all bacte-

ria can actively disperse from a biofilm (Petrova & Sauer, 2016). The detached bacteria then colonise other parts of the biofilm, making the biofilm a highly dynamic structure undergoing continuous remodelling (Flemming et al., 2016).

So far, the picture of biofilm formation is one of peaceful coexsistence, collaboration, and even neighbourly interspecies actions. A basis for this cooperation is increased overall benefits to the communities (Rendueles & Ghigo, 2015). However, competition between bacteria still exists within the biofilm. The metabolic by-products produced by some bacteria may be toxic for others, allowing the producers to gain a competitive advantage. The aforementioned acid-production by some bacteria can cause unfavourable conditions for species that prefer more neutral pH environments, particularly in the absence of the secondary feeders that would normally neutralise these compounds. A more direct example of bacterial competition is the ability of bacteria to produce substances that are toxic to other bacteria. These are often proteins or peptides termed bacteriocins, and can either inhibit or even kill other bacteria (Daw & Falkiner, 1996; Graham et al., 2017). *S. sanguinis* and *S. gordonii* can produce H_2O_2 that is toxic to *S. mutans*, a member of their own genus. *S. mutans* can, in turn, produce mutacin, which inhibits the growth of *S. sorbrinus*. There is no love lost among these close relatives (Chen et al., 1999). In addition to H_2O_2, oral streptococci can produce lactate by consuming carbohydrates, giving them a competitive advantage over acid-sensitive species by altering the local environment. Some species are resistant to specific metabolic by-products that others consider toxic, and may even consider them a delicacy (so to speak). *Veillonella* spp. are an example of organisms that thrive under these conditions, allowing both streptococci and *Veillonella* spp. to accumulate in the biofilm and create a favourable environment to select species (Edlund et al., 2018). These are simplistic examples, and often competition involves more interactions between multiple species taking on various roles of 'sensing', 'mediating', and 'killing' (Rendueles & Ghigo, 2015). Competition between and within species will ultimately shape the wider biofilm communities.

2.1.2 Dental calculus

The exact mechanism of dental calculus formation is not fully understood, but involves processes of biomineralisation and crystal formation within dental plaque. The main mineral components of calculus are crystals containing various combinations of calcium and phosphate ions. Other salts are also present, but the bulk of the crystals are made up of calcium phosphates. Initial mineralisation of dental plaque is a chemical process in which equilibrium of minerals in saliva and gingival crevicular fluid tips towards saturation with regard to calcium and phosphate, causing an increase of precipitation relative to dissolution. This means that when the concentration of ions increases and tips the balance between dissolution and precipitation, salts will accumulate within and on the surface of the biofilm. An increase in concentration of minerals within the biofilm reaches a critical threshold (supersaturation) and nucleation is triggered within the plaque matrix, initiating crystal growth. This may or may not involve spontaneous (or homogenous) nucleation, as it's unclear whether mineral concentrations are sufficient to cause spontaneous nucleation, or whether other biochemical processes act as a catalyst (Omelon et al., 2013). That it's a chemical process can be shown by the ability to produce calculus deposits in germ-free rats (Glas & Krasse, 1962; Theilade et al., 1964). However, it's unclear how the germ-free calculus compares to conventional calculus, and, to my knowledge there have only been studies on rats. Just because calculus growth can be induced in sterile conditions doesn't mean bacteria are not an essential part of the process. Bacteria are inevitably part of the scaffolding of dental calculus in humans, since, as I mentioned in the beginning of this chapter, our mouths are full of bacteria, and dental plaque is essentially built by bacteria. Mineralisation does seem to start in the biofilm matrix between microorganisms, but they are eventually also mineralised along with the biofilm matrix (Friskopp, 1983). There are pockets of living bacteria within dental calculus. These pockets and the layer of plaque that covers the surface of dental calculus are likely what cause the correlation between calculus presence and periodontal disease (B. T. K. Tan et al., 2004). While the process can be explained by chemistry, the conditions leading

up to and surrounding the process are both chemical and biological in nature, and certainly involve bacteria.

The main source of minerals in the oral cavity is saliva, which enters the mouth through salivary glands. The three main paired glands are the parotid, sublingual, and submandibular glands, located by the cheeks, under the tongue, and under the lower jaw bone, respectively. Saliva contains sodium (Na), potassium (K), calcium (Ca), chlorine (Cl), bicarbonate (buffer), and inorganic phosphate (Pi) (Dawes, 1970; Dodds et al., 2005), and the locations of the glands contribute to the pattern of dental calculus deposits within the mouth, which commonly grow on the buccal portion of maxillary (upper) molars and the lingual portion of mandibular (lower) incisors (Jin & Yip, 2002; White, 1997). Salivary pH also affects saturation of salts, which in turn is influenced by salivary flow rates. Increased flow rate of saliva will increase salivary pH, which reduces dissolution and increases precipitation of calcium and phosphate. This is an important mechanism that protects our teeth against demineralisation of the enamel caused by caries. Protection is provided by the exchange of calcium and phosphate from saliva to enamel (Dahlén et al., 2010). Saliva further acts as a buffer for the oral cavity, reducing the impact of short-term drops in pH caused by metabolic byproducts of acid-producing bacteria (Dodds et al., 2005; Jin & Yip, 2002). Higher rates of salivary flow are also likely to contribute to an increase in calcium and phosphate secretion in addition to pH, all contributing to an environment favouring plaque mineralisation. Metabolic byproducts produced by bacteria can also affect local pH, both pushing towards alkaline conditions as well as acidic. A major cause of acidic pH is metabolism of overabundant dietary sugars and starch, especially the metabolic activity of *Streptococcus mutans*, known to be one of the main culprits behind dental caries (Bowen et al., 2018; Duarte et al., 2008; Exterkate et al., 2010).

Conversely, alkaline conditions can be generated by metabolism of various products that can either be directly or indirectly linked to diet. One such product is urea. Urea is present in saliva, and its concentration depends on multiple fac-

tors. One of these factors is a high-protein diet, which increases levels of urea in serum and saliva (Lieverse, 1999). Hydrolysis of urea produces ammonia and causes a rise in pH. Bacteria possess the ability to produce ammonia from urea, which is further used by ammonia-oxidising organisms and converted to nitrite (Flemming et al., 2016; Sissons et al., 1994; Wong et al., 2002). In a similar way, arginine can be broken down to ammonia and increase in pH. Another pathway to alkalinity is through enzymatic activity. Saliva contains proteases which specialise in breaking down proteins into smaller components such as ammonia, and increased protease activity in saliva may therefore cause an increase in calculus production (Jin & Yip, 2002).

There are also a number of inhibitors and promoters of mineralisation present in the oral cavity, originating both from saliva and bacteria. Substances known to promote plaque mineralisation through hydroxyapatite formation and deposition, calcium-phospholipid-phosphate complexes (CPLX), are present in bacteria. *Corynebacterium matruchotii* (formerly *Bacterionema matruchotii*) accumulates calcium within its cell structure, and has therefore received a lot of attention in biomineralisation studies Ennever & Creamer (1967). Biomineralisation is not a feature unique to *Corynebacterium matruchotii*. Even species associated with caries may induce calcification under the right conditions and after cell death (Moorer et al., 1993; Sidaway, 1978). Inhibitors of biomineralisation include salivary proline-rich polypeptides, small amino acids important for the immune system; and statherin, a protein that controls the precipitation of calcium phosphate in saliva (Jin & Yip, 2002).

It's likely that multiple biomineralisation events occur under various conditions, resulting in a heterogeneous calculus composition with crystals of various stages of growth (Friskopp, 1983; Friskopp & Hammarström, 1980). The differing susceptibility of bacteria to calcification is also a contributor to the heterogeneous composition. Overall, plaque mineralisation is a complex interaction between conditions in the local environment, availability of minerals, the equilibrium between precipitation and dissolution, balance between nucleation

promoters and inhibitors.

2.2 Oral biofilm models

Biofilm models are a way of studying the growth and development of biofilms. By creating models that replicate the conditions and complexity (to some extent) of biofilms in a lab, models allow researchers to conduct various experiments to test the efficacy of treatments on the growth and pathogenicity of biofilms. There are many choices to be made when growing a biofilm, such as the composition of the initial oral microbial community, nutrient content and availability, and the makeup of the atmosphere in which the model is situated. As such, biofilm models can differ widely in their complexity and ability to mimic conditions in a human mouth. A choice of model can be made based on the end-goals of the research, or in some cases the choice is made for you based on (a lack of) available equipment and financial constraints. All models must have a defined biome containing a substratum and nutrients. The substratum is a surface on which the biofilm is intended to form and grow. For oral biofilm models the environment is the oral cavity and the substrata are the teeth, tongue, mucosa, or whatever the model is the biofilm supposed to be mimicking. The simplest models generally involve multiwell plates (e.g., 6-, 24-, and 98-well plates) with a substratum, usually glass cover-slips or hydroxyapatite discs, placed at the bottom of the well. Similar models suspend the substrata from a lid to promote active attachment of bacteria to the substrata (Exterkate et al., 2010). When the substrata are attached to a lid instead of the multiwell plates, it allows samples to be periodically transferred between solutions/media if necessary, adding more flexibility to the experimental setup.

Next, an inoculate is chosen. This can be anything from a single species of bacterium (pure culture), to multiple select species (defined consortium), to all organisms occurring naturally within a system (microcosm) (McBain, 2009). The purpose of the inoculate is to initiate biofilm formation by allowing the bacteria

to adsorb to the substrata, ideally in the presence of a conditioning film, such as saliva. For pure cultures and defined consortia, the inoculate may come from saliva or another oral site, such as dental plaque. The bacteria of interest are then isolated using selective media, essentially providing ideal growing conditions to certain types of bacteria, promoting their growth and eliminating others (e.g. Basson & van Wyk, 1996). Alternatively, the bacteria can be acquired directly from companies like the American Type Culture Collection (ATCC). For microcosms, the inoculate is often the saliva itself, or dental plaque, in its (mostly) raw form. The inoculate is added to the wells to initiate biofilm formation on the substrata as described above. As such, the content of the inoculate influences the complexity of the biofilm microbiome as well as the interactions between the communities within the biofilm (Røder et al., 2016). It's not always possible to use donated saliva as a growth medium for the duration of the experiment, especially if the experiment lasts more than a few days. Media with salivary components can be created as a substitute for long lasting experiments. There are many different recipes for media floating around out there, but most of them are generally a mixture containing mucin, proteins, minerals commonly found in saliva, and a buffer to maintain pH (Exterkate et al., 2010; Pratten et al., 1998; Shellis, 1978; Sissons et al., 1991; Tian et al., 2010).

More complicated models make use of increasingly sophisticated equipment to mimic the oral environment. Another level of model complexity can be added by adjusting the rate at which nutrients are dispersed through the system, and the overall nutrient supply. Nutrient distribution can be continuous, semi-continuous, or batch cultures, with the latter providing a finite amount of nutrients in a closed system. An example of a batch culture model is a biofilm grown on an agar plate, which has a finite amount of resources (Kearns et al., 2005). Once the nutrients in the agar have been depleted, that's it. At the other end of the spectrum is a system with a pump attached to a reservoir that can continuously supply the biofilm with growth medium, similar to salivary flow. In between the former options is the semi-continuous supply of nutrients. This can, for example, be the multiwell plate model with a lid, where the samples can be pe-

riodically transferred to new plates containing fresh growth medium (Exterkate et al., 2010). Other parameters that can be controlled to more closely simulate conditions in the oral cavity are pH and gas phase, as can be done with the multistation artificial mouth. This system gives researchers control over a large number of parameters using multiple chambers with complete control over the flow of treatment and/or nutrient conditions—environmental conditions such as pH, temperature, and gas phase—and access to real-time measurements (Sissons, 1997).

The duration of an experiment depends on the scope of the study. If the purpose is to learn more about initial biofilm formation and prevention, it may only be necessary to grow the biofilms for a few hours to 48 hours (Dibdin, 1981; Exterkate et al., 2010). If, instead, the goal is to learn more about biofilm maturation and calcification, the experiments can run for days or even weeks (Filoche et al., 2007; Sissons et al., 1991; Wong et al., 2002).

Models developed for studying oral biofilms include, in increasing complexity, the ACTA active attachment model (Exterkate et al., 2010), Calgary biofilm device (Ceri et al., 1999), modified Robbins device (Honraet & Nelis, 2006), constant depth film-fermenter (Peters & Wimpenny, 1988), and the multistation artificial mouth (Sissons et al., 1991) representing the upper echelon of complexity. Summaries of biofilm models, including benefits and limitations of the various types, can be found in reviews by McBain -McBain (2009), Tan and colleagues -C. H. Tan et al. (2017), and Røder and colleagues -Røder et al. (2016).

It might be tempting to think that the goal should always be to mimic the oral environment as closely as possible. However, there are benefits to more simplistic models, as well as limitations to the more sophisticated models. Benefits of pure cultures and defined consortia are reproducibility between experiments and more control over physiological and factors and making it easier to take various measurements. Microcosms have the benefit of more closely mimicking the complexity of the organisms' natural environment (McBain, 2009). However, even microcosms can be limited in their ability to recreate the complexity and di-

versity of the oral microbiome (Tian et al., 2010). Alternatives to *in vitro* models are *in situ* models which usually involve growing plaque on a removable surface inside the mouth of a willing participant. These models add a level of realism, as they are grown inside an actual oral cavity, and can reflect biogeographical differences in biofilm composition caused by differing conditions across the oral cavity. They also come with additional difficulties and reduced control over experimental parameters (Marsh, 1995; Zero, 1995).

Reiterating a point made in the Introduction, and Discussion, and probably somewhere in the articles as well, the benefit of using an oral biofilm model over naturally occurring dental calculus in the mouth of a research participant, is the control that it provides to tweak every aspect of the system, from the quantity and quality of nutrients available, to the amount of enzymes and bacterial species present. Plus, the added ethical benefit of not needing to ask someone to give up their oral hygiene regime for a few weeks. The following chapters, Chapter 3 and Chapter 4, provide a small glimpse of what a model looks like, and how it might be used to inform archaeological research.

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3 | Article 1

Assessing the validity of a calcifying oral biofilm model as a suitable proxy for dental calculus

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3.1 Introduction

Dental calculus is becoming an increasingly popular substance for exploring health and diet in past populations (Warinner et al., 2015). During life, dental plaque undergoes periodic mineralisation, trapping biomolecules and microfossils that are embedded within the dental plaque biofilm in the newly-formed dental calculus. This process is repeated as new plaque is deposited and subsequently mineralises, resulting in a layered structure representing a temporal record of biofilm growth and development (Warinner et al., 2014). The calculus serves as a protective casing for the entrapped biomolecules and microfossils, preserving them for thousands of years after death and burial (Fellows Yates et al., 2021). Studies using archaeological dental calculus span a wide range of topics in different regions and time periods. These include characterisation of the oral microbiome and its evolution in past populations (Adler et al., 2013; Fellows Yates et al., 2021; Kazarina et al., 2021; Velsko et al., 2019; Warinner et al., 2014), as well as extraction of microbotanical remains (Hardy et al., 2009; Henry & Piperno, 2008; Ma et al., 2022; Mickleburgh & Pagán-Jiménez, 2012) and other residues to infer dietary patterns and nicotine use (Bartholdy et al., 2023; Buckley et al., 2014; Eerkens et al., 2018; Hendy et al., 2018; Velsko, Overmyer, et al., 2017). Dental calculus has already provided a unique and valuable insight into the past, but the exact mechanism of the incorporation, retention, and preservation of microfossils and biomolecules exogenous to the microbial biofilm is largely unknown; even the process of plaque mineralisation is not fully understood (Jin & Yip, 2002; Omelon et al., 2013). This means that there may be hidden biases affecting our interpretations of dietary/activity patterns extrapolated from ancient dental calculus. These biases have been explored archaeologically (Fagernäs et al., 2022; Tromp et al., 2017) as well as in contemporary humans (Leonard et al., 2015) and non-human primates (Power et al., 2015), but not experimentally.

Dental plaque is an oral biofilm and is part of the normal state of the oral cavity. However, when left unchecked, plaque can lead to infections, such as den-

tal caries and periodontitis, and/or mineralisation (Marsh, 2006). The dental plaque biofilm grows in a well-characterized manner before mineralisation, in a process that repeats regularly to build up dental calculus. Shortly after teeth are cleaned (whether mechanically or otherwise), salivary components adsorb to the crown or root and form the acquired dental pellicle. The pellicle provides a viable surface for bacteria to attach, especially early-coloniser species within the genera *Streptococcus* and *Actinomyces* (Marsh, 2006). Once the tooth surface has been populated by specialists in surface-attachment, other species of bacteria can attach to the adherent cells, increasing the biofilm density and diversity. The bacterial species secrete polysaccharides, proteins, lipids, and nucleic acids, into their immediate environment to form a matrix that provides structural support, nutrition, and allows for environmental niche partitioning (Flemming et al., 2016).

Biofilms can become susceptible to calcification under certain microenvironmental conditions, including an increased concentration of salts and a decrease in statherin and proline-rich proteins in saliva, rises in local plaque pH, and increased hydrolysis of urea (White, 1997; Wong et al., 2002). These conditions can cause increased precipitation and decreased dissolution of calcium phosphate salts within saliva and the plaque biofilm. The resulting supersaturation of calcium phosphate salts is the main driver of biofilm mineralisation (Jin & Yip, 2002). The primary minerals in dental calculus are hydroxyapatite, octacalcium phosphate, whitlockite, and brushite. During initial mineralisation the main mineral component is brushite, which shifts to hydroxyapatite in more mature dental calculus (Hayashizaki et al., 2008; Jin & Yip, 2002). The exact elemental composition of dental calculus varies among individuals due to various factors, including diet (Hayashizaki et al., 2008; Ji et al., 2000).

Dental plaque can also be grown *in vitro*, and these oral biofilm models are commonly used in dental research to assess the efficacy of certain treatments on dental pathogens (Exterkate et al., 2010; Filoche et al., 2007) without the ethical issues of inducing plaque accumulation in study participants and the complex-

ity of access and sampling in humans or animals. Oral biofilm models are often short-term models grown over a few days, but longer term models also exist (up to six weeks) which are used to develop mature plaque or dental calculus (Middleton, 1965; Sissons et al., 1991; Velsko & Shaddox, 2018; Wong et al., 2002). A well-known limitation of biofilm models is the difficulty in capturing the diversity and complexity of bacterial communities and metabolic dependencies, microenvironments, nutrient availability, and host immune-responses in the natural oral biome (Bjarnsholt et al., 2013; Edlund et al., 2018; Velsko, Cruz-Almeida, et al., 2017; Velsko & Shaddox, 2018). These limitations can be overcome by complex experimental setups, but at the cost of lower throughput and increased requirements for laboratory facilities.

Despite the limitations, oral biofilm models have many benefits over *in situ* research. There are many variables involved in dental calculus formation, such as intra- and inter-individual variation in salivary flow, oral pH, and amylase activity, which can be hard to tease apart *in situ*. Oral biofilm models provide a controlled environment to explore the effect of selected variables on the growth of calculus and the retention of dietary components in the biofilm, as well as a means to identify how the methods used in archaeology may inadvertently bias the interpretations. This type of research has, so far, been limited, but has the potential to greatly benefit archaeological research on past diet (Radini & Nikita, 2022).

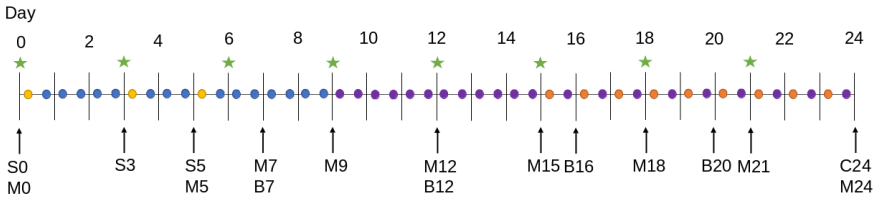
We present an oral biofilm model that can serve as a viable proxy for dental calculus for archaeology-oriented research questions. It is a multispecies biofilm using whole saliva as the inoculate, with a simple multiwell plate setup that is accessible even to smaller lab budgets and those with limited facilities for microbiology work. Here, we used next-generation sequencing and metagenomic classification to characterise the bacterial composition of our model dental calculus and compare it to oral reference samples, including saliva, buccal mucosa, plaque, and modern human dental calculus. This was done to ensure that the model microbiome is predominantly oral and not overgrown by environmental

contaminants. We then determined the mineral composition of the model dental calculus using Fourier transform infrared (FTIR) spectroscopy to verify the presence of calculus-specific mineral phases and functional groups, and perform a qualitative comparison with modern and archaeological reference calculus. Overall the model calculus is chemically similar to natural calculus, and has a predominantly oral microbiome. The microbial diversity and richness within the model samples were lower than oral reference samples, suggesting that the model samples do not contain identical species composition and abundances as the natural samples. The mineral composition closely resembles modern and archaeological reference calculus, predominantly comprised of carbonate hydroxyapatite with a similar level of crystallinity and order. As such, the model dental calculus presented here is a viable proxy to natural dental calculus and can be used to explore many of the currently unexplained processes we see in the archaeological material, when working within the limitations of an oral biofilm model.

3.2 Materials and methods

Our biofilm setup consists of whole saliva as the inoculate to approximate natural microbial communities within the human oral cavity, and a 24-well plate to generate multiple replicated conditions in a single experimental run (see Figure 3.1 for an overview of the protocol). The biofilm is grown for 25 days to allow time for growth of larger deposits and mineralisation. Raw potato and wheat starch solutions were added during the biofilm growth to explore the biases involved in their incorporation and extraction from dental calculus. These results are presented in a separate article (Bartholdy & Henry, 2022).

To determine the composition of microbial communities, we sampled the medium from the biofilm wells over the course of the experiment. We sequenced the DNA to identify species that are present in the model, and assess whether these mimic natural oral communities. During a separate experimental run, un-



S = saliva samples
M = artificial saliva samples
M0 = artificial saliva stock solution
B = biofilm samples
C = final plaque sample

- inoculate (whole saliva)
- Sucrose
- Sucrose + starch
- CPMU
- ★ Full media replacement

Layout of treatment conditions in the 24-well plate

	1	2	3	4	5	6
A	Potato	Potato	Potato	Potato	Potato	Potato
B	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat
C	Potato + Wheat	Potato + Wheat	Potato + Wheat	Potato + Wheat	Potato + Wheat	Potato + Wheat
D	Control	Control	Control	Control	Control	Control

Figure 3.1 – Overview of the protocol for biofilm growth. The samples for metagenomic analysis were grown in a separate experimental plate than the FTIR samples under the same experimental conditions. Biofilm (B) and calculus (C) samples were used for FTIR spectroscopy, and saliva (S), artificial saliva (M), and calculus samples were used for metagenomic analysis.

der the same conditions, we directly sampled the biofilms on multiple days and determined the mineral composition using FTIR, and compared the spectra to those of natural dental calculus, both modern and archaeological. Samples were taken from both controls and starch treatments, but differences between these samples were not explored in this study.

3.2.1 Biofilm growth

We employ a multispecies oral biofilm model following a modified protocol from Sissons and colleagues (1991) and Shellis (1978). The setup comprises a polypropylene 24 deepwell PCR plate (KingFisher 97000350) with a lid containing 24 pegs (substrata), which are autoclaved at 120°C, 1 bar overpressure, for 20 mins.

The artificial saliva (hereafter referred to as medium) is a modified version of the basal medium mucin (BMM) described by Sissons and colleagues (1991). It is a complex medium containing 2.5 g/l partially purified mucin from porcine stomach (Type III, Sigma M1778), 5 g/l trypticase peptone (Roth 2363.1), 10 g/l proteose peptone (Oxoid LP0085), 5 g/l yeast extract (BD 211921), 2.5 g/l KCl, 0.35 g/l NaCl, 1.8 mmol/l $CaCl_2$, 5.2 mmol/l Na_2HPO_4 (Sissons et al., 1991), 6.4 mmol/l $NaHCO_3$ (Shellis, 1978), 2.5 mg/l haemin. This is subsequently adjusted to pH 7 with NaOH pellets and stirring, autoclaved (15 min, 120°C, 1 bar overpressure), and supplemented with 5.8 (mu)mol/l menadione, 5 mmol/l urea, and 1 mmol/l arginine (Sissons et al., 1991).

Fresh whole saliva (WS) for inoculation was provided by a 31-year-old male donor with no history of caries, who abstained from oral hygiene for 24 hours, and no food was consumed two hours prior to donation. No antibiotics were taken up to six months prior to donation. Saliva was stimulated by chewing on parafilm, then filtered through a bleach-sterilised nylon cloth to remove particulates. Substrata were inoculated with 1 ml/well of a two-fold dilution of WS in sterilised 20% glycerine for four hours at 36°C, to allow attachment of the salivary pellicle and plaque-forming bacteria. After initial inoculation, the substrata were transferred

to a new plate containing 1 ml/well medium and incubated at 36°C, with gentle motion at 30 rpm. The inoculation process was repeated on days 3 and 5 by transferring the samples to a new plate with inoculate. Medium was partially refreshed once per day, by topping up the wells to the original volume with more medium, and fully refreshed every three days, throughout the experiment, by transferring the substrata to a new plate containing medium. To feed the bacteria, the substrata were transferred to a new plate, containing 5% (w/v) sucrose, for six minutes twice daily, except on inoculation days (days 0, 3, and 5), where the samples only received one sucrose treatment after inoculation.

On day 9, starch treatments were introduced, replacing sucrose treatments (except for control sample). As with the sucrose treatments, starch treatments occurred twice per day for six minutes, and involved transferring the substrata to a new plate containing a 0.25% (w/v) starch from potato (Roth 9441.1) solution, a 0.25% (w/v) starch from wheat (Sigma S5127) solution, and a 0.5% (w/v) mixture of equal concentrations (w/v) wheat and potato. All starch solutions were created in a 5% (w/v) sucrose solution. Before transferring biofilm samples to the starch treatments, the starch plates were agitated to keep the starches in suspension in the solutions, and during treatments, the rpm was increased to 60. The purpose of starch treatments was to explore the incorporation of starch granules into the model calculus. Starch treatments were initiated on day 9 (Figure 3.1) to avoid starch granule counts being affected by α-amylase hydrolysis from the inoculation saliva. An α-amylase assay conducted on samples from days 3, 6, 8, 9, 10, 12, and 14 also showed that there was no host salivary α-amylase activity in the system. The results of the starch incorporation and α-amylase activity assay have been reported in a separate article (Bartholdy & Henry, 2022).

After 15 days, mineralisation was encouraged with a calcium phosphate monofluorophosphate urea (CPMU) solution containing 20 mmol/l $CaCl_2$, 12 mmol/l NaH_2PO_4, 5 mmol/l Na_2PO_3F, 500 mmol/l urea (Pearce & Sissons, 1987; Sissons et al., 1991), and 0.04 g/l MgCl. The substrata were submerged in 1 ml/well

CPMU five times daily, every two hours, for six minutes, at 30 rpm. During the mineralisation period, starch treatments were reduced to once per day, two hours after the last CPMU treatment. This cycle was repeated for 10 days until the end of the experiment on day 24 (Figure 3.1). More detailed protocols are available at https://dx.doi.org/10.17504/protocols.io.dm6gpj9rdgzp/v1.

All laboratory work was conducted in sterile conditions under a laminar flow hood to prevent starch and bacterial contamination. Starch-free control samples that were only fed sucrose were included to detect starch contamination.

3.2.2 Metagenomics

Table 3.1 – Number of samples taken during the experiment, separated by sampling day and sample type.

Sample type	Sampling day	n
saliva	0	1
saliva	3	1
saliva	5	1
medium	5	2
medium	7	2
medium	9	2
medium	12	2
medium	15	2
medium	18	2
medium	21	2
medium	24	2
model_calculus	24	16

A total of 35 samples were taken during the experiment from the donated saliva, artificial saliva, and from the biofilm end-product on day 24 (Table 3.1). DNA extraction was performed at the Max Planck Institute for the Science of Human

History (Jena, Germany), using the DNeasy PowerSoil Kit from QIAGEN. C2 inhibitor removal step skipped, going directly to C3 step.

The DNA was sheared to 500bp through sonication with a Covaris M220 Focused-ultrasonicator. Double-stranded libraries were prepared (Aron et al., 2020) and dual indexed (Stahl et al., 2020), with the indexing protocol being adapted for longer DNA fragments. Briefly, the modifications consisted of adding 3 µl of DMSO to the indexing reaction, and extending the amplification cycles to 95°C for 60 s, 58°C for 60 s, and 72°C for 90 s. The libraries were paired-end sequenced on a NextSeq 500 to 150bp, and demultiplexed by an in-house script.

3.2.2.1 Preprocessing

The raw DNA reads were preprocessed using the nf-core/eager, v2.4.4 pipeline (Fellows Yates et al., 2020). The pipeline included adapter removal and read merging using AdapterRemoval, v2.3.2 (Schubert et al., 2016). Merged reads were mapped to the human reference genome (GRCh38) using BWA, v0.7.17-r1188 (Li & Durbin, 2009) (-n 0.01; -l 32), and unmapped reads were extracted using Samtools, v1.12. The final step of the pipeline, metagenomic classification, was conducted in kraken, v2.1.2 (Wood et al., 2019) using the Standard 60GB database (https://genome-idx.s3.amazonaws.com/kraken/k2_standard_20220926.tar.gz).

Environmental reference samples were downloaded directly from ENA and from NCBI using the SRA Toolkit. Oral reference samples were downloaded from the Human Metagenome Project (HMP), and modern calculus samples from Velsko et al. (2017). From the HMP data, only paired reads were processed, singletons were removed. *In vitro* biofilm model samples from Edlund et al. (2018) were used as a reference. Links to the specific sequences are included in the metadata. Human-filtered reads produced in this study were uploaded to ENA under accession number PRJEB61886.

3.2.2.2 Authentication

Species with lower than 0.001% relative abundance across all samples were removed from the species table. SourceTracker2 (Knights et al., 2011) was used to estimate source composition of the abundance-filtered oral biofilm model samples using a Bayesian framework, and samples falling below 70% oral source were removed from downstream analyses. Well-preserved abundance-filtered samples were compared to oral and environmental controls to detect potential external contamination. The R package decontam v1.22.0 (Davis et al., 2018) was used to identify potential contaminants in the abundance-filtered table using DNA concentrations with a probability threshold of 0.95 and negative controls with a probability threshold of 0.05. Putative contaminant species were filtered out of the OTU tables for all downstream analyses.

3.2.2.3 Community composition

Relative abundances of communities were calculated at the species- and genus-level, as recommended for compositional data (Gloor et al., 2017). Shannon index and Pileou's evenness index were calculated on species-level OTU tables of all model and oral reference samples using the vegan v2.6.4 R package (Oksanen et al., 2022). Shannon index was calculated for all experimental samples to see if there is an overall loss or gain in diversity and richness across the experiment. Sparse principal component analysis (sPCA) was performed on model biofilm samples to assess differences in microbial composition between samples within the experiment, and a separate sPCA analysis was performed on model calculus and oral reference samples. The sPCA analysis was conducted using the mixOmics v 6.26.0 R package (Rohart et al., 2017).

The core microbiome was calculated by taking the mean genus-level relative abundance within each sample type for model calculus, modern reference calculus, sub- and supragingival plaque. Genera present at lower than 5% relative abundance were grouped into the category 'other'. Information on the oxygen tolerance of bacterial species was downloaded from BacDive (Reimer et al.,

2022) and all variations of the major categories anaerobe, facultative anaerobe, and aerobe were combined into the appropriate major category. At the time of writing, 55.7% species were missing aerotolerance values. This was mitigated by aggregating genus-level tolerances to species with missing values, and may have some errors (although unlikely to make any significant difference).

3.2.2.4 Differential abundance

Differential abundance of species was calculated using the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) method from the ANCOMBC R package v2.4.0 (Lin & Peddada, 2020), with a species-level OTU table as input. Results are presented as the log fold change of species between paired sample types with 95% confidence intervals. P-values are adjusted using the false discovery rate (FDR) method. Samples are grouped by sample type (i.e. saliva, plaque, modern calculus, model calculus). To supplement the sPCA analyses, we visualised the log-fold change of the top 30 species in each of principal components 1 and 2, allowing us to see which species are enriched in the different samples and causing clustering in the sPCA.

3.2.3 FTIR

To determine the mineral composition and level of crystallisation of the model dental calculus samples, we used Fourier Transform Infrared (FTIR) spectroscopy. We compared the spectra of model dental calculus with spectra of archaeological and modern dental calculus and used a built-in Omnic search library for mineral identification (Mentzer et al., 2014; Weiner, 2010b). The archaeological dental calculus was sampled from an isolated permanent tooth from Middenbeemster, a rural, 19th century Dutch site (Lemmers et al., 2013). Samples were analysed at the Laboratory for Sedimentary Archaeology, Haifa University. The analysis was conducted with a Thermo Scientific Nicolet is5 spectrometer in transmission, at 4 cm^{-1} resolution, with an average of 32 scans between 4000 and 400 cm^{-1} wavenumbers.

Table 3.2 – Summary of samples used in FTIR analysis, including type of sample, sampling day, number of samples (n), and mean weight in mg.

Sample type	Sampling day	n	Weight (mg)
biofilm	7	2	0.79
biofilm	12	3	1.01
biofilm	16	7	2.00
biofilm	20	6	3.50
model_calculus	24	8	3.87

Analysis was conducted on 26 model calculus samples from days 7, 12, 16, 20, and 24 (Table 3.2). Some samples from the same sampling day had to be combined to provide enough material for analysis. Samples analysed with FTIR were grown during a separate experimental run from the samples sequenced for DNA, but following the same setup and protocol (as described above). Samples were analysed following the method presented in Asscher, Regev, et al. (2011) and Asscher, Weiner, et al. (2011). A few μg of each sample were repeatedly ground together with KBr and pressed in a 7 mm die under two tons of pressure using a Specac mini-pellet press (Specac Ltd., GS01152). Repeated measurements of the splitting factor (SF) of the absorbance bands at 605 and 567 cm–1 wavenumbers were taken after each grind, and a grind curve was produced following Asscher, Regev, et al. (2011) to try and detect changes in the hydroxyapatite crystallinity over time. Samples were ground and analysed up to six times (sample suffix a-f) for the grinding curve. Grinding curves were prepared for samples from days 16, 20, and 24. No grind curves were produced for samples from days 7 and 12. These were largely composed of organics and proteins, and did not form enough mineral (hydroxyapatite) for analysis. The splitting factor of carbonate hydroxyapatite was calculated using a macro script, following Weiner & Bar-Yosef (1990). The calculation involves dividing the sum of the height of the absorptions at 603 cm^{-1} and 567 cm^{-1} by the height of the valley between

them. Following Asscher, Regev, et al. (2011) and Asscher, Weiner, et al. (2011), we plotted the splitting factor against the full width at half maximum (FWHM) of the main absorption at 1035-1043 cm^{-1} to explore crystallinity (crystal size) and the order and disorder of hydroxyapatite. We then compared our grinding curve slopes and FWHM to the ones produced by Asscher, Weiner, et al. (2011). Asscher, Weiner, et al. (2011) and Asscher, Regev, et al. (2011) demonstrated that while the decrease in FWHM of each grinding in the curve reflects a decrease in particle size due to grinding, the location of the curves within a plot of the FWHM against the splitting factor expresses the disorder effect. Thus the curves with steeper slopes, higher splitting factor, and lower FWHM represent lower levels of disorder in the mineral (Figure 2 in Asscher, Weiner, et al., 2011).

3.2.4 Statistics

Statistical analysis was conducted in R version 4.3.3 (2024-02-29) (Angel Food Cake) (R Core Team, 2020). Data cleaning and wrangling performed with packages from tidyverse (Wickham et al., 2019). Plots were created using ggplot2 v3.4.4 (Wickham, 2016).

3.3 Results

3.3.1 Metagenomic analysis

3.3.1.1 Sample authentication

To determine the extent of contamination in our samples, we performed a source-tracking analysis using SourceTracker2 (Knights et al., 2011). Results suggest that the majority of taxa across samples have an oral microbial signature, and therefore our samples are minimally affected by external contamination (Figure S1). We compared SourceTracker2 results to a database of oral taxa from the cuperdec v1.1.0 R package (Fellows Yates et al., 2021) to prevent removal of samples where oral taxa were assigned to a non-oral source (Figure

S2), as some taxa with a signature from multiple sources are often classified as "Unknown" (Velsko et al., 2019). We included several oral sources, which may increase the risk of this occurring. Samples containing a large proportion (>70%) of environmental contamination were removed. The removed samples were predominantly medium samples from later in the experiment, and a few model calculus samples. After contaminated samples were removed, suspected contaminant-species were removed from the remaining samples using the decontam R package (Davis et al., 2018). After contamination removal, samples consisted of between 88 and 284 species with a mean of 182.

3.3.1.2 Decrease in community diversity across experiment

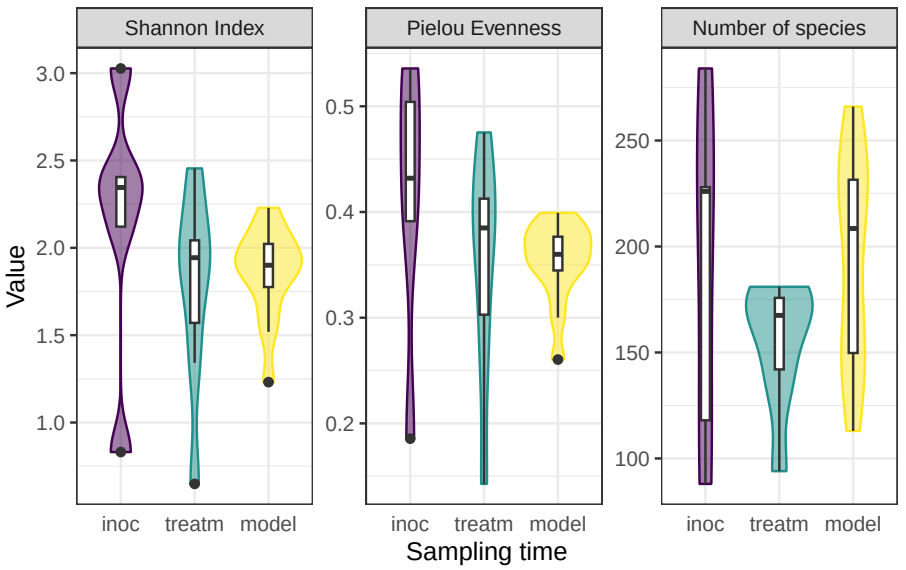


Figure 3.2 – Plot of Shannon Index, Pielou Evenness Index, and number of species across experiment samples grouped by sampling time. inoc = samples from days 0-5; treatm = samples from days 6-23; model = model calculus samples from day 24.

To monitor the development of microbial communities over the course of the experiment, we used the Shannon Index to assess the species diversity and richness at various stages of our protocol. Samples were grouped into sampling categories due to low sample sizes on sampling days (inoc = days 0, 3, 5; treatm = days 7, 9, 12, 15; model = day 24). There was a slight decrease in mean Shannon Index between inoculation and treatment samples, followed by a slight increase to model calculus samples, as well as a decrease in variance within sample types. The Pielou Evenness Index showed a similar pattern while the number of species increased between the treatment period and the final model calculus (Figure 3.2).

3.3.1.3 Medium and model calculus samples are distinct from the inoculate

We next examined whether there is a change in the species composition over time in our samples by assessing the beta-diversity in a PCA. The species profiles of the saliva inoculate used in our experiment were distinct from both medium and model calculus samples. Most of the separation of saliva from model calculus is on PC1 of the sPCA, where most of the positive sample loadings are driven by anaerobic species (model calculus), especially *Selenomonas* spp, and negative loadings are predominantly facultative anaerobes and some aerobes, such as *Rothia* and *Neisseria* spp (saliva). Medium and saliva are separated mostly on PC2, with medium samples located between saliva and model calculus samples. Model calculus samples also cluster separately from the medium samples on PC2, with some overlap between the more mature medium samples and model calculus. Most of the negative loadings separating saliva and model calculus from medium samples are dominated by *Actinomyces* spp., while positive species loadings are more diverse, and seemingly unrelated to aerotolerance (Figure 3.3).

We determined whether there are species that are differentially abundant between our sample types using the ANCOMBC R package (Lin & Peddada, 2020),

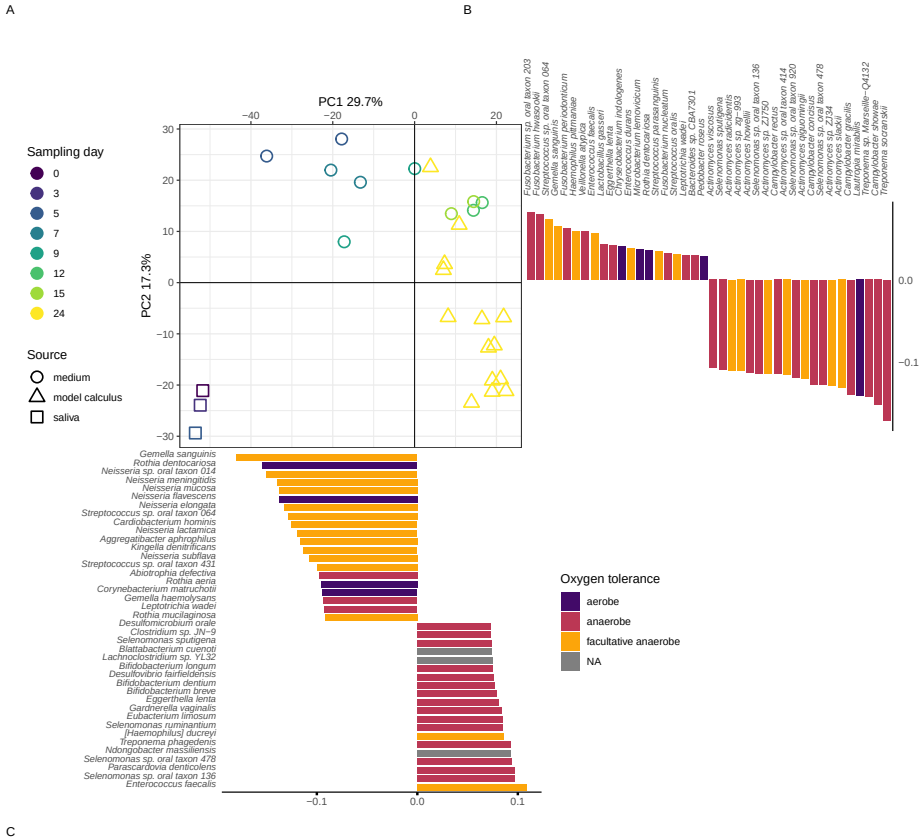


Figure 3.3 – sPCA on species-level counts and oxygen tolerance in samples from this study only. Figure shows the main sPCA plot (A), species loadings on PC2 (B), and species loadings on PC1 (C).

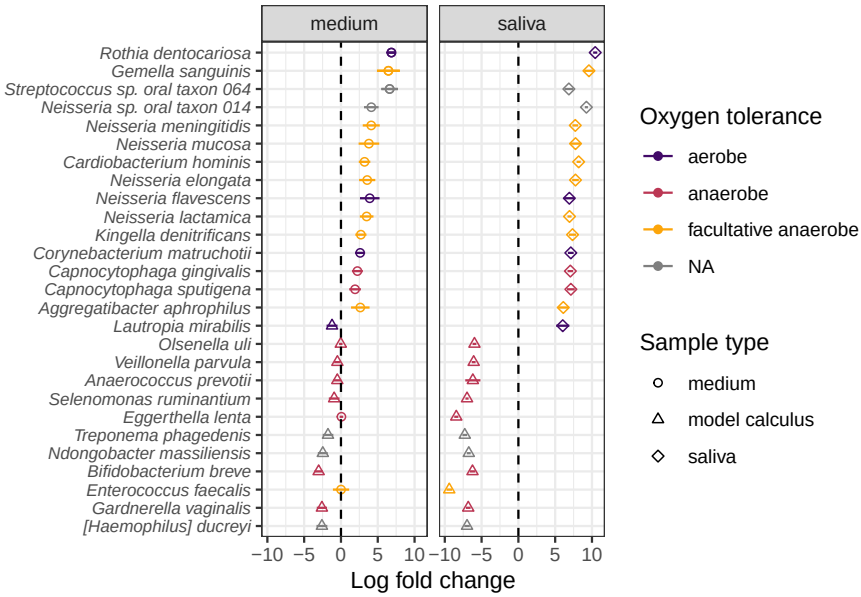


Figure 3.4 – Log-fold changes between sample types. Circles are species enriched in the medium, triangles are enriched in model calculus, and diamonds are enriched in saliva. Lines are standard error. Plot shows the top 30 absolute log-fold changes between model calculus and saliva.

giving us an idea of how the biofilm develops under our experimental conditions. Species enriched in saliva compared to model calculus are largely aerobic or facultatively anaerobic, while species enriched in model calculus compared to saliva are mainly anaerobes. The differences between saliva and calculus are more pronounced than between medium and model calculus, which is expected (Figure 3.4).

3.3.1.4 Lower diversity in artificial samples than oral references

We used the Shannon Index to compare alpha-diversity in our model to oral reference samples. The mean Shannon Index of model samples—medium, model calculus, reference *in vitro* biofilm were consistently lower than the means of oral reference samples—mucosa, modern reference dental calculus, saliva, and subgingival and subgingival plaque. The Pielou species evenness index has a similar distribution, although the comparative biofilm samples have a higher mean than biofilm samples from this study. Saliva inoculate samples from this study have a lower mean Shannon index than reference samples, which may have contributed to the lower alpha-diversity in model samples compared to reference samples. The number of species follows the same trend.

3.3.1.5 Model calculus is distinct from dental calculus and other oral samples

We calculated the mean relative abundances of the genera in each sample to compare the core genera of model calculus with oral reference samples. The most common genera (>5% relative abundance) are shown in Figure 3.6. The main overlap between the model calculus and oral reference samples is the high relative abundance of *Streptococcus*. Model calculus consists mostly of *Enterococcus* and *Veillonella* spp., despite both having low abundance in donor saliva. *Enterococcus* are also known environmental contaminants, and we cannot exclude environmental contamination as a possible source for these species in our model. Oral reference samples have a more balanced composition, as they

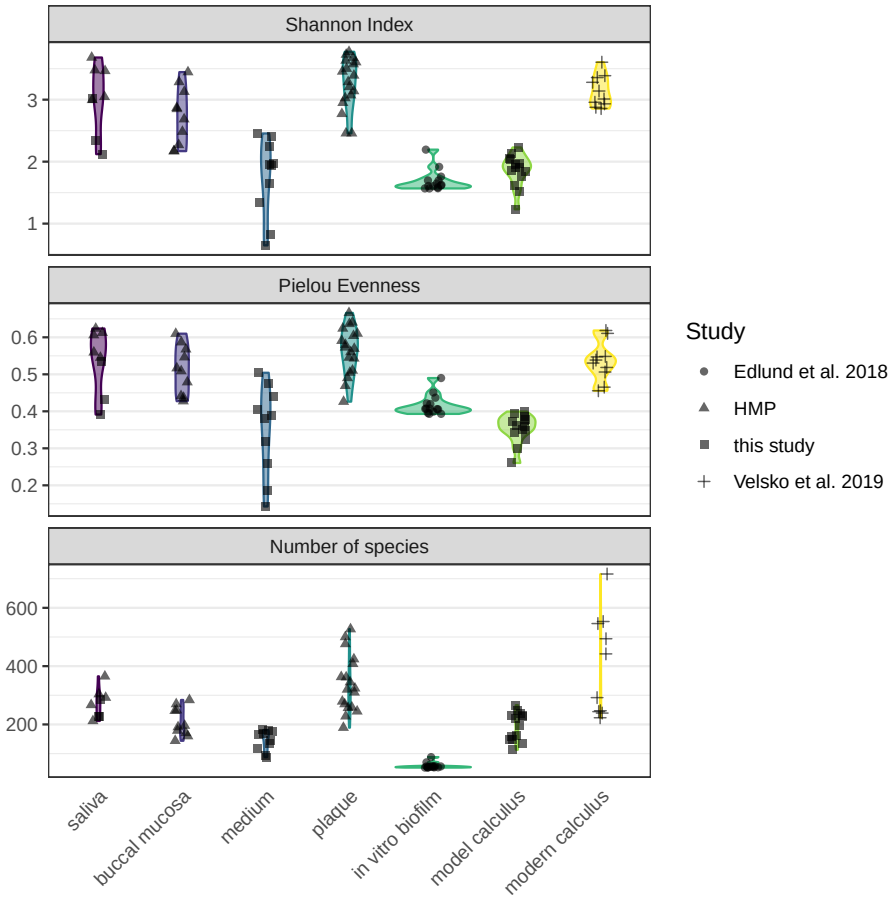


Figure 3.5 – Shannon Index for model calculus and medium samples, as well as oral reference samples and comparative *in vitro* study.

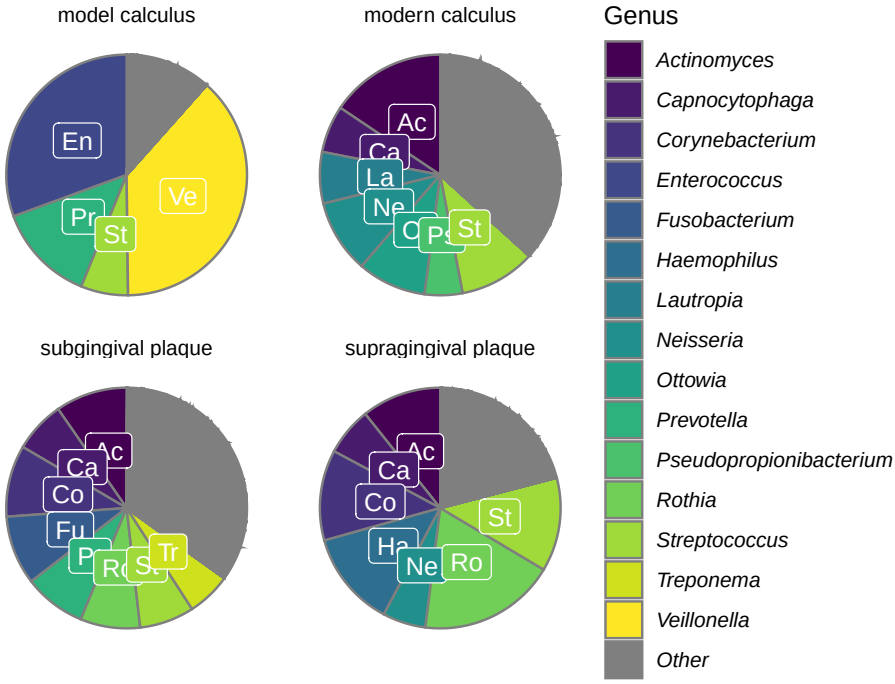


Figure 3.6 – Core genera within the different types of samples represented as mean relative abundances at the genus level. Other = other genera present in lower than 5% relative abundance.

are also represented by fastidious early-coloniser species like *Capnocytophaga* and *Neisseria* spp., which require an environment with at least 5% carbon dioxide to thrive (Tønjum & van Putten, 2017).

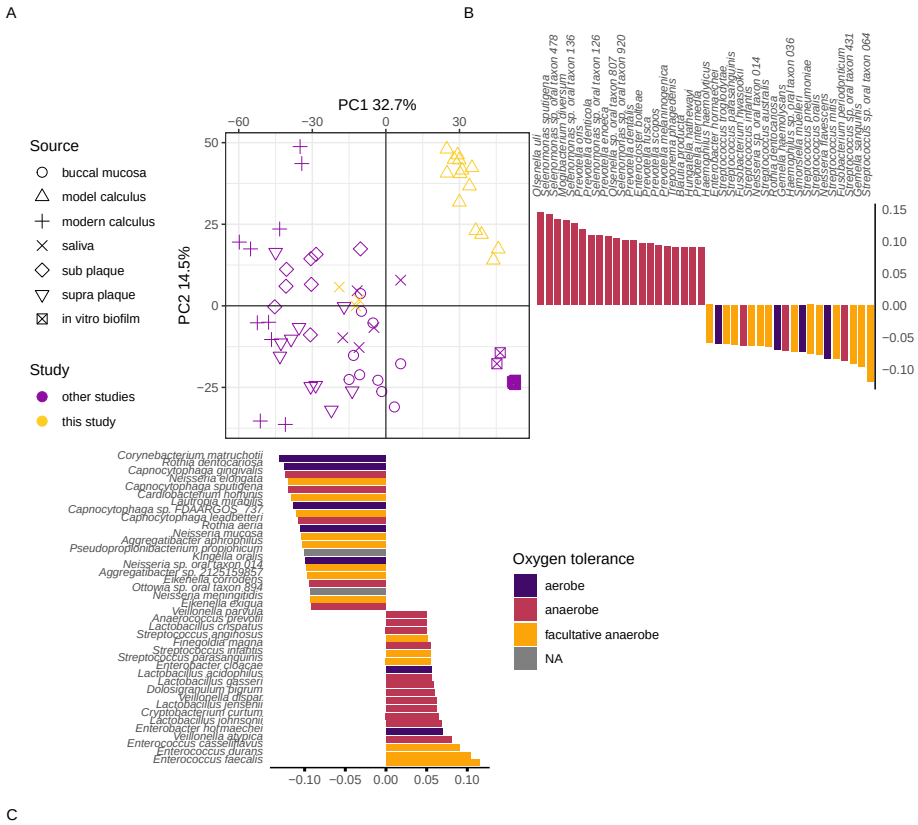


Figure 3.7 – sPCA on species-level counts from model calculus and reference samples. Figure shows (A) the main sPCA plot, (B) the species loadings from PC2, and (C) species loadings on PC1.

To directly compare the beta-diversity of our model calculus with oral reference samples, including modern dental calculus, we used an sPCA including only our model calculus and reference samples. Model calculus samples are distinct

from both the oral reference samples and the biofilm model reference samples. They are separated from oral reference samples mainly on PC1, and from biofilm model reference samples (and, to some extent, oral samples) on PC2. The highest negative contributions are a mix of all types of aerotolerance, while the positive contributions are mostly (facultative) anaerobes, with *Enterococcus* spp. as the top three positive contributors to PC1. Top negative contributors are *Capnocytophaga* spp as well as the aerobes *Corynebacterium matruchotii* and *Rothia dentocariosa*. The top positive contributors to PC2 are all anaerobes, mainly from the genus *Selenomonas*. Top negative contributors to PC2 are a mix of aerotolerances, with many *Streptococcus* spp (Figure 3.7).

To investigate which species are enriched in different sample types, and compare the final product of our model with naturally occurring plaque and calculus samples, we performed differential abundance analysis on our model calculus samples, modern dental calculus, and sub- and supragingival plaque. Based on the differential abundance analysis the main differences between model calculus and oral reference samples, when looking at the top 30 contributors to PC1, are that the oral reference samples are enriched with species with a diverse oxygen tolerance from a wide range of genera, while the model calculus is enriched with *Enterococcus* spp. The largest differences occur in *Corynebacterium matruchotii*, *Rothia dentocariosa*, and *Capnocytophaga gingivalis* (Figure 3.8A). This is echoed when looking at the top 30 contributors to PC2, where most of the species are enriched in model calculus, all of which are anaerobes, and the largest differences occurring in *Cryptobacterium curtum*, *Eggerthella lenta*, and *Mogibacterium diversum* (Figure 3.8B).

3.3.2 Samples show an increased mineralisation over the course of the experiment

To determine whether the model dental calculus is comparable to natural dental calculus, both modern and archaeological dental calculus were analysed with FTIR spectroscopy to ascertain their composition.

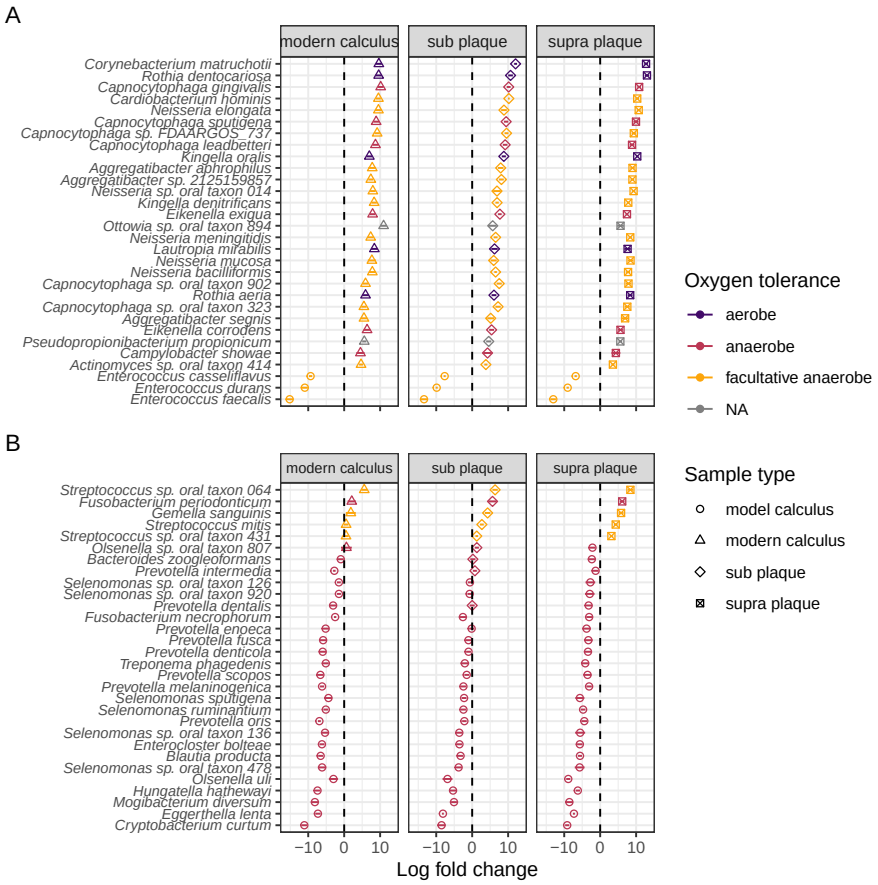


Figure 3.8 – Log-fold changes between sample types. Circles are species enriched in the model calculus, triangles in modern calculus, diamonds are enriched in subgingival plaque, and squares in supragingival plaque. Plot shows the top 30 loadings (absolute value) in PC1 (A) and PC2 (B) between model calculus and other sample types, ordered by decreasing log-fold change. Bars represent standard error.

It is evident that between days 7 and 24 there is a decrease of the protein components and increase of the inorganic mineral carbonate hydroxyapatite. The model calculus samples from the end of the experiment are similar to both the modern and archaeological reference samples. The main difference is a lower organic component in reference samples seen as a reduced amide I peak at around 1637 compared to the carbonate peak at around 1420, and an absence of amide II and III. Further, there is a reduction in CH3 bands at 3000-2900 cm^{-1} (Figure 3.9A-D).

Sample spectra from days 7 and 12 are characterised by a high content of proteins as evident by the strong amide I absorbance band at 1650, a less pronounced amide II band at 1545 cm^{-1}, and the small amide III band at 1237 cm^{-1}. Related to the organic component of the samples are also the three marked CH_3 and CH_2 stretching vibrations at 2960, 2920, and 2850 cm^{-1} wavenumbers. The presence of mineral component is evident from the presence of C–O_3^{2-} absorbance bands at 1450 and 1400 cm^{-1} wavenumbers typical of carbonates, and P–O_4^{3-} absorbance band at 1080 and 1056 cm^{-1} which are related to phosphate minerals. There is a large variation between the spectra, possibly indicating different formation rates of the different components in the samples (Figure 3.9A and B).

In spectra from days 16 to 24, the ratio of amides to PO_4 has shifted, with the main peak shifting to the PO_4 v_3 absorbance band at 1039–1040 cm^{-1}, indicating that the main component of the samples is carbonate hydroxyapatite. A well-defined PO_4 doublet at 600 and 560 is present. Small CO_3^{2-} asymmetric stretching at 1450 cm^{-1} and 1415 cm^{-1}, and stretching vibrations at 875-870 cm^{-1} indicate that the carbonate minerals component is also becoming more crystallised. There is a decreased variability between the spectra, with most spectra exhibiting a higher phosphate-to-protein/lipid ratio (Figure 3.9C and D).

3.3.3 Model calculus has a similar mineral composition to natural calculus

Archaeological and modern reference spectra are largely indistinguishable and consist of a broad O–H absorbance band (3400 cm^{-1}) related to amid a and hydroxyl group, weak CH3 bands (3000–2900 cm^{-1}), amide I band (1650 cm^{-1}) which is related to the protein content, carbonate (1420, 1458-1450, 875-870 cm^{-1}), and phosphates (1036-1040, 602-4, 563-566 cm^{-1}) (Figure 3.9E) which, together with the hydroxyl and the carbonate, can be identified as derived from carbonate hydroxyapatite, the main mineral found in mature dental calculus (Hayashizaki et al., 2008; Jin & Yip, 2002).

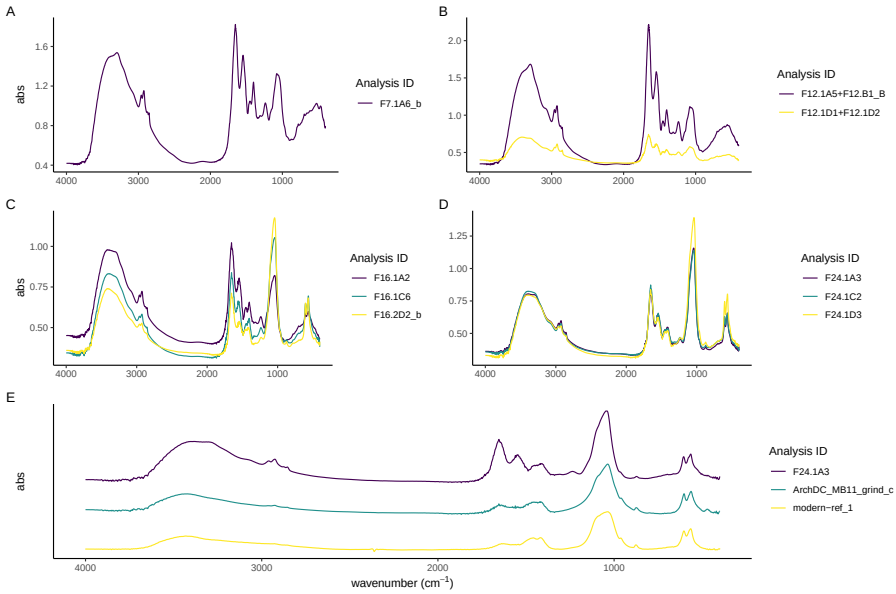


Figure 3.9 – Select spectra from all experiment sampling days; (A) day 7, (B) day 12, (C) day 16, and (D) day 24. Absorbance bands in stretching mode around 3400 cm–1 typical of the hydroxyl group. Analysis ID for model samples is constructed as: F[day sampled].[well sampled]_[grind sample].

3.3.4 Samples show similar crystallinity and order to reference calculus

We determined the level of crystallinity and order of the carbonate hydroxyapatite in our samples as an indication for its maturity by using the grinding curves method presented by Asscher, Regev, et al. (2011) and Asscher, Weiner, et al. (2011).
Samples were compared to published trendlines for archaeological and modern enamel (Asscher, Regev, et al., 2011). We see no appreciable differences between days 16, 20, and 24. The archaeological dental calculus shows a slightly increased slope compared to model calculus from the three sampling days used in the grind curve (Figure 3.10), possibly indicating larger crystal size due to more complete crystalisation. The steeper slope of enamel samples is consistent with a more ordered structure in enamel compared to dental calculus.

3.4 Discussion

In this study we present a calcifying oral biofilm model to produce artificial dental calculus. Our proposed use of the model is to address a variety of research questions related to dietary information extracted from dental calculus, in both modern and archaeological samples. For that to be feasible, the model needs to serve as a viable proxy to dental calculus grown under natural conditions, i.e., in the human oral cavity. It needs, as much as possible, to mimic the diversity and complexity of the natural oral microbiome, while also offering control over factors such as dietary input, growth conditions, and replicability within and between experiments. Here, we assessed the viability of our model as a proxy for dental calculus using metagenomic classification and FTIR analysis to explore the bacterial and mineral composition, and compare with oral reference samples.

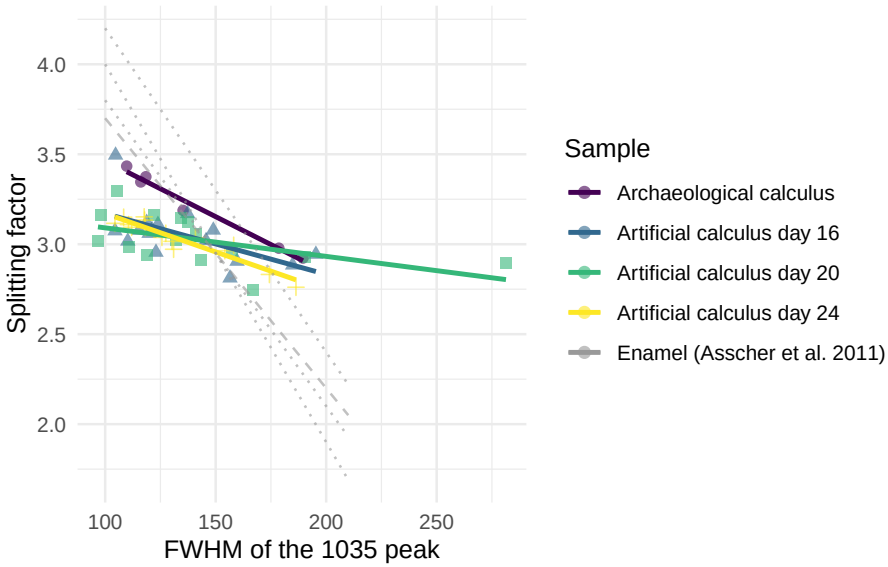


Figure 3.10 – Grinding curves of our biofilm and model calculus compared to published trendlines (dashed light grey lines) for archaeological (dotted line) and modern (dashed line) enamel.

3.4.1 Microbiome

Model calculus has lower species diversity than inocula saliva and oral reference samples, which is a common limitation in biofilm models (Bjarnsholt et al., 2013; Edlund et al., 2013). The donated saliva for the experiment had a lower diversity than the reference saliva samples, and may have contributed to a lower diversity in experimental samples. Consequently, there is also a lower diversity and richness when compared to other modern oral reference samples, including oral mucosa, saliva, plaque, and calculus. Samples of the medium from early in the experiment have similar species profiles to the donated saliva, but gradually diverge over the course of the experiment. This may be caused by experimental setup not sufficiently mimicking the oral environment, allowing species to thrive that do not normally thrive in the natural oral environment.

Oral reference samples have a relative abundance of streptococci similar to our model, but a more diverse representation from other genera and an overall higher species diversity and richness than our model. Reference samples also had a more diverse aerotolerance profile than our model, which primarily consisted of (faculatative) anaerobes. Species within predominantly aerobic genera, are deficient in the model, suggesting a shift from a largely aerotolerant profile to an anaerobic profile during the experiment. While our model is not set up as an anaerobic system, the anaerobes seem to have outcompeted aerobes and, to some extent, facultative anaerobes. This is likely a result of communities of bacteria within the biofilm creating favourable microenvironments facilitated by the protective properties of the biofilm matrix (Edlund et al., 2018; Flemming et al., 2016).

Overall, the majority of model calculus samples contained a distinctly oral signature, providing a promising starting point for the use of the model as a viable proxy to dental calculus. The main differences between model and oral reference samples may be due to human variation, as there can be large differences in the oral microbiome of two individuals at the species level due to variations in age, sex, and other demographic factors, as well as how and when saliva sam-

ples were collected (Burcham et al., 2020; Nearing et al., 2020). Whether or not distinct microbial profiles, and the extracellular matrix they produce, will affect the retention of dietary particles in plaque remains to be seen, but is an important question to address in the future.

3.4.2 Mineralisation

FTIR analysis allowed us to address the mineralisation process of the model, which showed an increasing mineral composition over the course of the experiment. As the model biofilm matured, the predominantly organic content of early samples was replaced by inorganic content in the form of carbonated hydroxyapatite, consistent with a shift from a high presence of bacterial cells in a matrix of extracellular polysaccharides (Jain et al., 2013; Sutherland, 2001; Zhang et al., 1998) to a predominantly mineral content.

The model calculus samples resemble both the modern reference calculus and the archaeological calculus in mineral composition and crystallinity. The steeper slope in the grind curve plots of the archaeological sample suggests that the crystals in archaeological samples are larger, and hence more ordered than in model calculus. A possible explanation is that the inorganic crystals within archaeological calculus have had more time to grow into the space left by degraded organic matter (Weiner, 2010a); however, we only analysed one archaeological sample and cannot definitively address this. The short duration of model calculus growth may also have affected the results, compared to the longer-term growth and mineralisation of natural calculus. The constant disruptions in growth of *in vivo* dental plaque/calculus, due to oral hygiene and other external pressures on biofilm growth, may lead to multiple stages of calcium phosphates, whereas our model has more stable growth conditions.

One of the most well-known biomineralisers, *Corynebacterium matruchotii* (Ennever et al., 1978; Takazoe et al., 1970), exhibited a lower abundance in our model calculus compared to modern reference calculus. However, the mineral composition of the end results were similar, reinforcing the idea that, under the right

circumstances, biofilms with a range of microbial profiles can facilitate mineralisation (Moorer et al., 1993). Bacteria and their ability to secrete an extracellular matrix are integral in the formation of dental calculus, and inevitably serve as part of the structure that dental calculus is built upon (Rohanizadeh & LeGeros, 2005), while the exact species composition of the biofilm communities may be less important.

3.4.3 Replicability

Model calculus displayed similar species diversity and microbial profiles across all samples, indicating a high level of replicability between samples in the experimental run. It remains to be seen whether the replicability within the experiment also scales up to between-experiment replicability in our model, though others have already shown that replicability in long-term models is possible when using the same inocula (Velsko & Shaddox, 2018). The variation in mineral composition in our model was initially high, but samples from day 24 were largely similar in composition as observed in the FTIR spectra. The use of a simple multiwell plate setup allows us to submit many samples to the same conditions, increasing replicability between samples (Exterkate et al., 2010).

3.4.4 Limitations

While our in vitro model calculus system provides reproducible and consistent artificial dental calculus for archaeological research, as demonstrated by the species composition and the mineralisation properties, we recognise the model has several limitations. Our single-donor approach may have affected the diversity of the model. The donated saliva from our study had a lower mean Shannon Index than other saliva samples. The lower diversity may be caused by only using one donor instead of pooling saliva from multiple individuals. However, having a single inoculum donor allows us to maintain the integrity of a native oral microbiome which may be lost when samples are pooled (Edlund et al., 2013). It is also possible that the diversity was affected by the collection and storage

methods we used. This has been shown to have minimal effect on microbial profiles at the genus level (Lim et al., 2017), but some effect on beta diversity calculations (Omori et al., 2021).

Some samples were grown with starch-sucrose solutions as nutrients, while controls were grown with sucrose only. Due to the financial cost, we did not sequence enough samples of each nutrient treatment to assess the influence of starch on the microbial community or mineral composition. Biofilms were grown in a standard shaking bacterial growth incubator, rather than an incubator specific to cell cultures. The lack of complex environmental control may cause the model to deviate from its natural growth over the 25 days that the experiment is run, due to a lack of precise control over conditions such as pH and salivary flow rates.

There is also the possibility that contamination was introduced into the model during the experiment. While the CPMU solution was prepared under sterile conditions, the solution itself was not autoclaved or filter-sterilised. In the species composition metagenomic analysis, all medium samples collected after the introduction of CPMU on day 14 were removed by the authentication step because the majority of species appeared to derive from environmental sources indicating external contamination. Going forward we recommend filter-sterilising solutions that are not autoclaved.

To avoid disturbing the growth and development of our biofilm, we took samples of media from the bottom of the wells after three days without full media replacement, careful not to disturb other plate-bound biofilms. The samples may therefore not fully reflect the composition of the biofilm itself. Going forward we recommend sampling from the actual biofilm, as this is the sample type under investigation.

3.4.5 Future work

Further protocol optimisation will also be necessary to address some of the limitations of our current model, such as reducing the frequency of medium replacement (currently every three days) to help promote the growth of slow-growing fastidious organisms and limit generalists such as enterococci, and supplementing it with serum to provide additional nutrients and biofilm stability (Ammann et al., 2012; Tian et al., 2010). More infrequent medium replacement would facilitate slow-growing bacteria in establishing their metabolic relationships, allowing the byproducts of some species to become abundant enough for others that depend on these to grow (Marsh, 2005).

Our goals for additional validation measures involve functional profiles of bacteria, to see if metabolic behaviour of bacteria is consistent with *in vivo* conditions, and whether this is affected by the presence/absence of amylase and starch treatments. The absence of host salivary α-amylase activity in our model (as shown in Bartholdy & Henry (2022)) provides an opportunity to explore the effect of various amylase levels on biofilm growth and composition, as well as the incorporation of dietary compounds in dental calculus.

The model can also be used to explore limitations and biases of methods used to reconstruct past dietary patterns from dental calculus. To this end, sucrose and raw starch treatments can be replaced with other dietary components of interest, such as cooked starches, whole plant extracts, and various proteins.

3.5 Conclusions

The bacterial profile of our model calculus is not an exact match to the natural modern or archaeological reference calculus, but species richness and diversity falls within a similar range as the reference *in vitro* model, and the core genera are predominantly oral. Our model calculus had a distinct microbial profile from modern reference calculus, but a similar mineral composition to modern and

archaeological reference calculus, consisting of carbonate hydroxyapatite and similar levels of crystallinity and order, with a slightly higher organic phase.

Our model has many potential benefits within archaeological research, especially since the setup does not require highly specialised equipment, making it accessible to many labs within the archaeological sciences. It can be used to test many fundamental aspects of the process of incorporation, retention, and subsequent extraction of various dietary components from archaeological dental calculus. Using an oral biofilm model in a controlled environment with known dietary input, we can learn more about how different methods of food processing in the past may affect results of dental calculus analyses, and how the methods we use may further distort this picture. Our method can be used to test methods (e.g. DNA, proteomics, etc.), decontamination protocols, as well as training on these methods and protocols without depleting limited archaeological resources. The purpose of our model is not to replace studies conducted on archaeological and natural dental calculus, but rather to balance limitations of each method and serve as a complementary approach to expand our toolkit.

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4 | Article 2

Investigating Biases Associated with Dietary Starch Incorporation and Retention with an Oral Biofilm Model

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4.1 Introduction

Dental calculus has proven to contain a wealth of dietary information in the form of plant microfossils (Hardy et al., 2009; Henry & Piperno, 2008), proteins (Hendy et al., 2018; Warinner, Hendy, et al., 2014), and other organic residues (Buckley et al., 2014). This dietary information can be preserved within the mineralised dental plaque over many millennia, providing a unique window into the food-related behaviours of past populations (Henry & Piperno, 2008; Jovanović et al., 2021; Tao et al., 2020) and extinct species (Hardy et al., 2012; Henry et al., 2014).

Until recently, only a few studies directly investigated the presence of plant microremains in the dental calculus of archaeological remains. The ability to extract phytoliths from the dental calculus of archaeological fauna to investigate diet was first noted by Armitage (1975), and later by Middleton and Rovner (1994), and Fox and colleagues (1996). Starches and phytoliths were extracted from human dental calculus by Cummings and Magennis (1997).
In more recent years, the study of dental calculus has increased exponentially, and the wealth of information contained within the mineralised matrix has largely been acknowledged. The use of dental calculus spans a wide variety of archaeological research areas, such as oral microbiome characterisation (including pathogens) through the analysis of DNA and proteins (Adler et al., 2013; Warinner, Rodrigues, et al., 2014), microbotanical remains (Hardy et al., 2009; Henry & Piperno, 2008; Mickleburgh & Pagán-Jiménez, 2012), other organic residues and proteins from dietary compounds (Buckley et al., 2014; Hendy et al., 2018), and nicotine use (Eerkens et al., 2018). Especially the extraction of starch granules has become a rich source of dietary information, as starch granules have proven to preserve well within dental calculus over a variety of geographical and temporal ranges (Henry et al., 2014; Jovanović et al., 2021; Piperno & Dillehay, 2008; Tao et al., 2020).

Despite this, our knowledge of dental calculus and the incorporation pathways

of the various markers is limited (Radini et al., 2017), as is our knowledge of information-loss caused by these pathways. Additionally, the methods we use to extract and analyse dental calculus, and make inferences on past diets represent another potential source of bias. Studies on both archaeological and modern individuals have explored these biases in more detail. Extraction methods were tested by Tromp and colleagues (2017), specifically regarding decalcification using HCl or EDTA. The authors found significantly more starches with the EDTA extraction method than the HCl extraction method; however, as noted by the authors, comparisons involving archaeological calculus are problematic due to variability between and within individuals. Studies conducted on modern humans (Leonard et al., 2015) and non-human primates (R. C. Power et al., 2015; Robert C. Power et al., 2021) have explored how well microremains (phytoliths and starches) extracted from dental calculus represent the actual dietary intake. These studies are justifiably limited, despite meticulous documentation and observation, due to unknown variables and uncertainty involved in this kind of *in vivo* research. Dental calculus is a complex oral biofilm with a multifactorial aetiology and variable formation rates both within and between individuals (Haffajee et al., 2009; Jepsen et al., 2011), contributing to the stochasticity of starch representation being observed in numerous studies. Additionally, the concentration of oral α-amylase differs both between and within individuals (Froehlich et al., 1987; Nater et al., 2005), causing different rates of hydrolysis of the starch granules present in the oral cavity. Add to this the effects of the many different methods of starch processing (Hardy et al., 2018), as well as post-depositional processes that are still being explored (García-Granero, 2020; Mercader et al., 2018), and it becomes clear that using dental calculus to reconstruct diet is a highly unpredictable process.

In this exploratory study, we use an oral biofilm model to investigate the retention of starch granules within dental calculus in a controlled laboratory setting, allowing us full control over dietary input. Our main questions concern the representation of granules extracted from the calculus compared to the actual intake. How much of the original diet is incorporated into the calculus, and how much is

recovered? Is there differential loss of information from specific dietary markers that affects the obtained dietary information, and how does this affect the representation of diet from extracted microremains?
We find that, despite the absence of α-amylase in the model, a limited proportion of the starch input is actually retained in the calculus. We also observed a shift in the size ratios of individual starch granules that are incorporated into the calculus, and that the number of incorporated starch granules increases as the size of the calculus deposit increases.

4.2 Materials and Methods

4.2.1 Biofilm formation

In this study we employ a multispecies oral biofilm model following a modified protocol from Sissons and colleagues (1991) and Shellis (1978). In brief, a biofilm inoculated with whole saliva was grown on a substrate suspended in artificial saliva, and fed with sugar (sucrose). After several days of growth, the biofilm was exposed to starch solutions. Mineralisation of the biofilm was aided by exposure to a calcium phosphate solution. After 25 days of growth, the mineralised biofilm was collected for further analysis. The setup comprises a polypropylene 24 deepwell PCR plate (KingFisher 97000351Ø) with a lid containing 24 pegs, which is autoclaved at 12Ø°C, 1 bar overpressure, for 2Ø mins. The individual pegs were the substrata on which the calculus grew. Using this system allowed for easy transfer of the growing biofilm between saliva, feeding solutions, and mineral solutions.

The artificial saliva (AS) is a modified version of the basal medium mucin (BMM) described by Sissons and colleagues (1991). It contains 2.5 g/l partially purified mucin from porcine stomach (Type III, Sigma M1778), 5 g/l trypticase peptone (Roth 2363.1), 1Ø g/l proteose peptone (Oxoid LP0085), 5 g/l yeast extract (BD 211921), 2.5 g/l KCl, Ø.35 g/l NaCl, 1.8 mmol/l $CaCl_2$, 5.2 mmol/l Na_2HPO_4 (Sissons et al., 1991), 6.4 mmol/l $NaHCO_3$ (Shellis, 1978), 2.5 mg/l haemin. This is

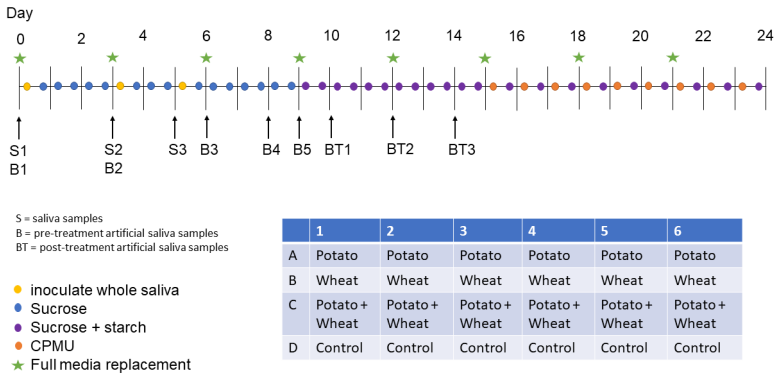
subsequently adjusted to pH 7 with NaOH pellets and stirring, autoclaved (15 min, 120°C, 1 bar overpressure), and supplemented with 5.8 μmol/l menadione, 5 mmol/l urea, and 1 mmol/l arginine (Sissons et al., 1991).

Fresh whole saliva (WS) for inoculation was provided by a 31-year-old male donor with no history of caries, who abstained from oral hygiene for 24 hours. No food was consumed two hours prior to donation and no antibiotics were taken up to six months prior to donation. The saliva was filtered through a sterilised (with sodium hypochlorite, 10–15% active chlorine) nylon cloth to remove particulates. Substrata were inoculated with 1 ml/well of a two-fold dilution of WS in sterilised 20% (v/v) glycerine for four hours at 36°C, to allow attachment of the salivary pellicle and plaque-forming bacteria. After initial inoculation, the substrata were transferred to a new plate containing 1 ml/well AS and incubated in a shaking incubator (Infors HT Ecotron) at 36°C, 30 rpm. The inoculation process was repeated on days 3 and 5. AS was partially refreshed once per day and fully refreshed every three days, throughout the experiment, by transferring the substrata to a new plate containing stock AS. To feed the bacteria, the substrata were transferred to a new plate, containing 5% (w/v) sucrose, for six minutes twice daily, except on inoculation days (days 0, 3, and 5), where the samples only received one sucrose treatment after inoculation.

Starch treatments were initiated on day 9 to avoid starch granule counts being affected by α-amylase hydrolysis from saliva inoculation days. An α-amylase (EC 3.2.1.1) activity assay was conducted to confirm that no amylase was present in the model before starch treatments started. Starch treatments replaced sucrose treatments, occurring twice per day for six minutes. The starch treatments involved transferring the substrata to a new plate containing a 0.25% (w/v) starch from potato (Roth 9441.1) solution, a 0.25% (w/v) starch from wheat (Sigma S5127) solution, and a 0.5% (w/v) mixture of equal concentrations (w/v) wheat and potato. All starch treatments were created in dH_2O with 5% (w/v) sucrose. Before transferring biofilm samples to the starch treatment plate, the plates were agitated to keep the starches in suspension in the solutions. Dur-

ing treatments, the rpm was increased to 60 to facilitate contact between starch granules and biofilms.

After 15 days, mineralisation was encouraged with a calcium phosphate monofluorophosphate urea (CPMU) solution containing 20 mmol/l $CaCl_2$, 12 mmol/l NaH_2PO_4, 5 mmol/l Na_2PO_3F, 500 mmol/l urea, and (0.04 g/l MgCl) (Pearce & Sissons, 1987; Sissons et al., 1991). The substrata were submerged in 1 ml/well CPMU for six minutes, five times daily, in a two-hour cycle. During the mineralisation period, starch treatments were reduced to once per day after the five CPMU treatments. This process was repeated for 10 days until the end of the experiment on day 24 (see Figure 4.1 for an overview of the protocol).



	1	2	3	4	5	6
A	Potato	Potato	Potato	Potato	Potato	Potato
B	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat
C	Potato + Wheat	Potato + Wheat	Potato + Wheat	Potato + Wheat	Potato + Wheat	Potato + Wheat
D	Control	Control	Control	Control	Control	Control

Figure 4.1 – Overview of experiment protocol including the plate setup.

All laboratory work was conducted in sterile conditions under a laminar flow hood to prevent starch and bacterial contamination. Control samples that only received sucrose as a treatment were included to detect starch contamination from the environment or cross-contamination from other wells in the same plate.

4.2.2 Amylase activity detection

An α-amylase (EC 3.2.1.1) activity assay was conducted on artificial saliva samples collected from the plate wells on days 3, 6, 8, 9, 10, 12, and 14. Whole saliva samples were collected on days 0, 3, and 5 as positive controls. Collected samples were stored at 4°C until the assay was conducted on day 18. All samples and standard curves were run in triplicates on two separate plates. Positive control saliva samples were compared against a standard curve containing H_2O, while artificial saliva samples were compared against a standard curve containing stock AS (due to the colour of artificial saliva). Two photometric readings were conducted for each plate with a 540 nm filter on a Multiskan FC Microplate Photometer (Thermo Scientific 51119000). The protocol is a modified version of an Enzymatic Assay of α-Amylase (https://www.sigmaaldrich.com/NL/en/technical-documents/protocol/protein-biology/enzyme-activity-assays/enzymatic-assay-of-a-amylase) (Bernfeld, 1955), which measures the amount of maltose released from starch by α-amylase activity. Results are reported in units (U) per mL enzyme, where 1 U releases 1 μmole of maltose in 6 minutes. The detailed protocol can be found here: https://www.protocols.io/view/amylase-activity-bw8jphun.

4.2.3 Treatment solutions

A 1 ml aliquot of each starch solution was taken, from which 10 μl was mounted on a microscope slide with an 18 x 18 mm coverslip, and counted under a light microscope (Zeiss Axioscope A1). For wheat and mixed treatment samples, we counted three slide transects (at ca. 1/4, 1/2, and 3/4 of the slide), and the sample counts were extrapolated to the total number of granules exposed to the samples over 16 days of treatments (see Supplementary Material for more details). For potato treatment samples, the whole slide was counted.

4.2.4 Extraction method

Extraction of starches from the calculus samples was performed by dissolving the calculus in 0.5 M ethylenediaminetetraacetic acid (EDTA) (Le Moyne & Crowther, 2021; Modi et al., 2020; Tromp et al., 2017), and vortexing for 3 days until the sample was completely dissolved. Twenty μl of sample was mounted onto a slide with an 18x18 mm coverslip. When transferring the sample to the slide, the sample was homogenised using the pipette to ensure that the counted transects were representative of the whole slide. The count from the slide was extrapolated to the whole sample (see Supplementary Material for more detail).

Both wheat and potato granules were divided into three size categories: small (<10 μm), medium (10 – 20 μm), and large (>20 μm).

4.2.5 Statistical analysis

Statistical analysis was conducted in R version 4.3.3 (2024-02-29) (R Core Team, 2020) and the following packages: tidyverse (Wickham et al., 2019), broom (Robinson et al., 2021), here (Müller, 2020), and patchwork (Pedersen, 2020).

To see if biofilm growth was differently affected by starch treatments, a one-way ANOVA with sample weight as the dependent variable (DV) and treatment as the grouping variable (GV) was conducted. To analyse granule counts and calculate size proportions, mean counts for each treatment were taken across both experimental plates, resulting in a mean count for each granule size category within each treatment.

Pearson's *r* was conducted on sample weight and total starch count, as well as sample weight and starch count per mg calculus. The total count for each sample within a treatment was standardised by z-score to account for the differences in magnitude between the potato and wheat counts. This was applied to total biofilm weight and starch count per mg calculus (also z-score standardised) to account for differences in starch concentration in the calculus (as per Wesolowski et al., 2010).

4.3 Results

All samples yielded sufficient biofilm growth and starch incorporation to be included in the analysis (Figure 4.2), resulting in a total of 48 biofilm samples (two plates of 24), 45 of which were used for analysis (three samples were set aside for later analysis). Most control samples contained no starch granules, while some contained negligible quantities (see Supplementary Material).

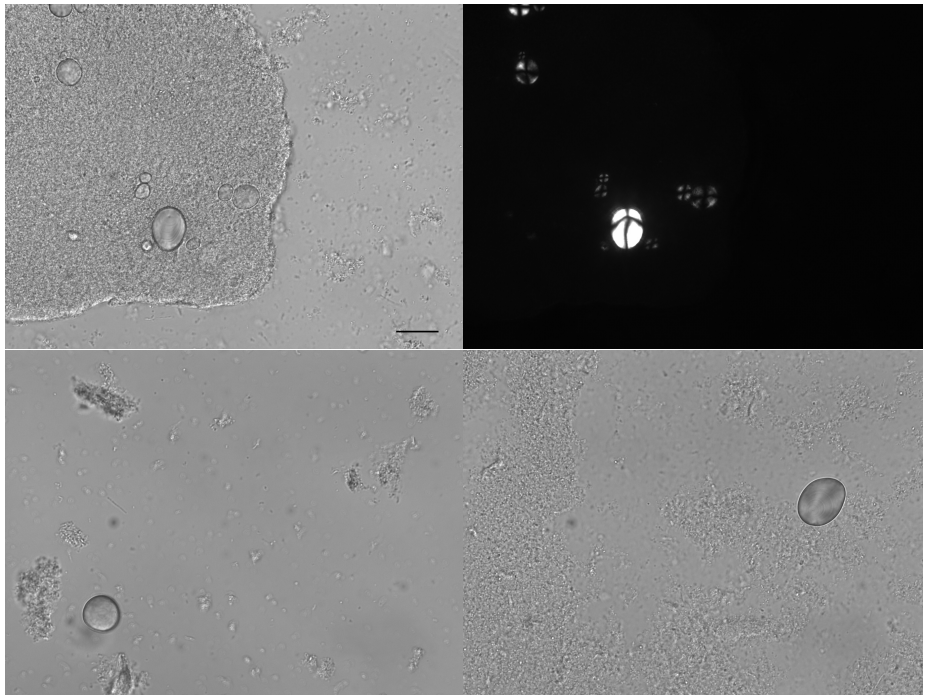


Figure 4.2 – Microscope images of biofilm samples that were exposed to the starch solutions. Starch granules can be seen within bacterial communities and isolated. Scale bar = 20 µm.

4.3.1 No amylase activity detected in the model

No α-amylase activity was detected in any of the artificial saliva samples from any of the days that were sampled. Only positive controls (saliva) contained amylase activity that could be detected in the assay, ranging from 9.93 to 30.2 U/mL enzyme (full results can be found in the Supplementary Material). The results are not comparable to other studies presenting α-amylase activity levels in humans, as the unit definition may differ; however, they are sufficient to show that there is no activity in the model.

4.3.2 Treatment type had minimal effect on biofilm growth

A one-way ANOVA suggests that the type of starch used during the biofilm growth period had a minimal effect on the growth of the biofilm (expressed as total dry weight of the sample), $F(3, 43) = 1.16$, $p = 0.335$. A summary of sample weights is available in Table 4.1.

Table 4.1 – Summary statistics for biofilm dry-weights (in mg) by treatment.

Treatment	Mean	SD	Min	Max
control	5.44	2.45	1.67	11.20
mix	4.28	1.95	1.50	8.44
potato	6.25	2.07	2.54	8.92
wheat	5.53	3.45	0.56	9.80

4.3.3 Starch counts

It was not possible to differentiate between potato and wheat starches smaller than ca. 10 μm. These were counted as wheat, as we assumed that the majority of the small granules were wheat. We make this assumption based on the counts

of small starches in the wheat-only and potato-only solutions. Of the combined amount of small starches in these two solutions, 99.2% are from wheat.

The separate wheat and potato solutions were made with a 0.25% (w/v) starch concentration, while the mixed-starch solution was made with 0.25% (w/v) of each starch, with a total concentration of 0.50% (w/v). The mixed treatment had the highest absolute count of starch granules in solution (mean = 2.9×10^7), while the biofilms exposed to the wheat solution preserved the greatest number of granules (mean = 2.77×10^4). The potato treatment had the lowest absolute counts in both the solution (3.02×10^6) and in the biofilm samples (4850) (Table 4.2 and Table 4.3).

Table 4.2 – Mean starch counts from solutions, including the proportional makeup of the different sizes of granules.

Solution	Starch	Small (%)	Medium (%)	Large (%)	Total (%)
mix	potato		1051733 (53.1%)	928000 (46.9%)	1979733 (100.0%)
mix	wheat	18838400 (69.7%)	6403200 (23.7%)	1794133 (6.6%)	27035733 (100.0%)
mix	both	18838400 (64.9%)	7454933 (25.7%)	2722133 (9.4%)	29015467 (100.0%)
potato	potato	123733 (4.1%)	1337867 (44.4%)	1554400 (51.5%)	3016000 (100.0%)
wheat	wheat	16139467 (63.5%)	6434133 (25.3%)	2830400 (11.1%)	25404000 (100.0%)

Table 4.3 – Mean starch counts extracted from samples with standard deviation (SD), including the proportion of granule sizes of the total count.

Treatment	Starch	Small (%)	SD	Medium (%)	SD	Large (%)	SD	Total (%)	SD
mix	potato			1959 (79.6%)	1801	501 (20.40%)	446	2460 (100%)	2189
mix	wheat	9515 (54.60%)	8860	6522 (37.4%)	6026	1381 (7.93%)	1196	17417 (100%)	15878
mix	both	9515 (47.90%)	8860	8480 (42.7%)	7653	1882 (9.47%)	1596	19877 (100%)	17768
potato	potato	351 (7.24%)	297	3565 (73.6%)	2402	930 (19.20%)	929	4846 (100%)	3316
wheat	wheat	15235 (55.00%)	11944	12148 (43.9%)	11052	1953 (7.06%)	2016	27680 (100%)	23554

4.3.3.1 Proportion of available starches incorporated in samples

The proportion of total starches from the solutions that were incorporated into the samples ranged from 0.06% to 0.16%, with potato granules being more readily incorporated than wheat in both the separated- and mixed-treatment samples (Table 4.4). There is an inverse relationship between the absolute starch count in the solutions and the proportional incorporation of starches in the biofilm samples, i.e., potato had the lowest absolute count in solutions, but the highest proportional incorporation, and vice versa for the mixed treatment.

Table 4.4 – The mean percentage of starches from the solutions that were incorporated into the samples.

Treatment	Starch	Small	Medium	Large	Total
mix	potato		0.19%	0.05%	0.12%
mix	wheat	0.05%	0.10%	0.08%	0.06%

Table 4.4 – The mean percentage of starches from the solutions that were incorporated into the samples.

Treatment	Starch	Small	Medium	Large	Total
mix	both	0.05%	0.11%	0.07%	0.07%
potato	potato	0.28%	0.27%	0.06%	0.16%
wheat	wheat	0.09%	0.19%	0.07%	0.12%

Wheat incorporation was most affected in the mixed-treatment samples, with only 0.06% of the total available starches being incorporated into the sample, compared to 0.16% in the separated wheat treatment.

4.3.3.2 Size ratios differ between solutions and samples

Overall, medium starch granules had a higher mean rate of incorporation (0.171%) than small (0.120%) and large (0.066%) starch granules across all treatments, while large potato starches had the lowest rate of incorporation across all treatments.

The difference in incorporation between the size categories resulted in a change in size ratios between the original starch solutions and the extracted samples. Large potato granules (> 20 μm) were most affected, with a 32.3% decrease in relative abundance in the potato-only treatment, and a 26.5% decrease in mixed treatments. Medium granules increased in relative abundance across all samples, while small granules decreased in wheat treatments and increased in potato treatments (Figure 4.3).

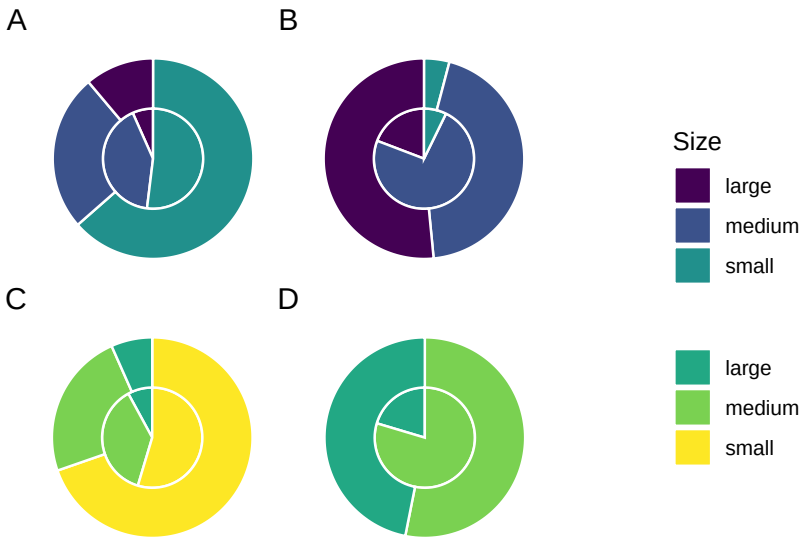


Figure 4.3 – Proportion of sizes of starch granules from solutions (outer ring) and treatment samples (inner ring) in separated wheat (A) and potato (B) treatments, and mixed wheat (C) and potato (D) treatments.

4.3.3.3 Biofilm weight correlated positively with extracted starch counts

Pearson's *r* suggests a strong positive correlation between the total weight of the biofilms and the total starch count (standardised by z-score) extracted from the samples across treatments, $r = 0.659$, 90%CI[0.463, 0.794], $p < 0.001$ (Figure 4.4A).

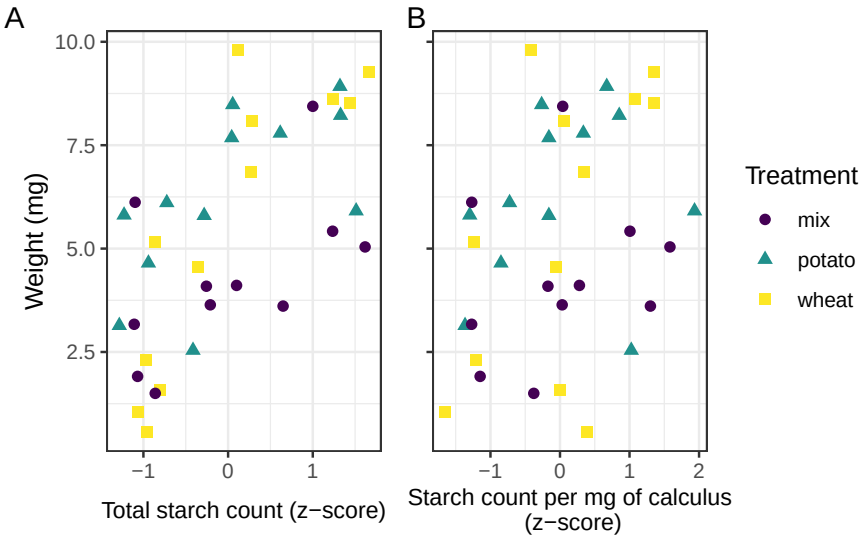


Figure 4.4 – Scatter plots of (A) sample weight in mg and standardised starch count by z-score for separated treatments, and (B) sample weight in mg and standardised count of starch grains per mg calculus.

The same test was applied to total biofilm weight and starch count per mg calculus (also standardised by z-score), resulting in a weak positive correlation, r = 0.3, 90%CI[0.0618, 0.506], p = 0.0403 (Figure 4.4B).

4.4 Discussion

Here, we have provided a method for exploring the incorporation of dietary starches into the mineral matrix of a dental calculus biofilm model. Our results show that a very low proportion of the starches exposed to the biofilm during growth are retained in the mineral matrix, and that the size of the starch granules may affect the likelihood of incorporation. The proportions of starch granules

(of all sizes) present in the extracted samples were similar across all treatments (0.06% to 0.16%), despite large differences in absolute granule counts between wheat (mean = 25,404,000) and potato (mean = 3,016,000) solutions.
The absolute counts, however, differed more visibly between treatments and was proportional with the total count of granules in the treatment solutions. Wheat and mixed solutions had the highest absolute mean count of starch granules, and also had the highest absolute mean count of starch granules extracted from the dental calculus (Table 4.2 and Table 4.3). This suggests that the starches that are more frequently consumed will be present in higher quantities in the dental calculus, at least prior to inhumation and degradation in the burial environment. Despite the low proportion of granules recovered from the model calculus (0.06% to 0.16%), the absolute counts were still substantially greater than counts recovered from archaeological remains (Tromp et al., 2017; Tromp & Dudgeon, 2015; Wesolowski et al., 2010), which could in part be due to the lack of oral amylase activity in our model. Previous research conducted on dental calculus from contemporary humans and non-human primates suggest a high level of stochasticity involved in the retention of starch granules in dental calculus, and that starch granules extracted from dental calculus are underrepresented with regard to actual starch intake, which is consistent with our findings (illustrated by high standard deviations and low proportional incorporation). Leonard and colleagues (2015) found individual calculus samples to be a poor predictor of diet in a population, as many of the consumed plants were missing from some individual samples, but were present in others.
Power and colleagues (2015) presented similar findings in non-human primates, where phytoliths were more representative of individual diets than starch granules. The size bias is also consistent with the findings by Power and colleagues (2015), who found that plants producing starches 10–20 μm in size were overrepresented; however, the representation of granules larger than 20 μm in their study is unclear.

We have also shown that the size of the starch granules influences the likelihood of incorporation into the calculus. Starch granules larger than 20 μm in

maximum length were underrepresented in the calculus samples compared to the original starch solutions, an effect that was consistent across all three treatments. Medium granules (10–20 μm) were often over-represented (Table 4.4, and Figure 4.3). Large potato granules were most affected, potentially because of the greater size-range. They can reach up to 100 μm in maximum length, whereas wheat granules generally only reach up to 35 μm (Gismondi et al., 2019; Haslam, 2004; Seidemann, 1966, pp. 174–176). Granule morphology may also play a role. Large wheat granules are lenticular and have a larger surface area compared to volume, whereas large potato granules are ovoid and have a larger volume compared to surface area (Jane et al., 1994; Reichert, 1913, pp. 364–365; Seidemann, 1966, pp. 174–176; van de Velde et al., 2002). Another potentially important factor from our results is the size of the calculus deposit. We found a strong positive correlation between size of biofilm deposit and retained starch granules (Figure 4.4A), meaning larger calculus deposits contain a higher quantity of granules; a result that contradicts findings from archaeological contexts (Dudgeon & Tromp, 2014; Wesolowski et al., 2010). When the concentration of starch granules per mg calculus is considered, the correlation is weaker, but still present (Figure 4.4B). While the larger deposits contain a higher absolute count, our findings also suggest that they contain a slightly higher concentration of starches. This may also explain the lower mean retention of starch granules in mixed treatments compared to wheat treatments. Wheat treatment samples (mean = 5.53 mg) were on average larger than mixed treatment samples (mean = 4.28 mg) (Table 4.1); and while mixed treatment solutions contained the highest mean overall granule counts, wheat treatment samples had the highest mean starch retention. Further research is needed to determine why this differs from previous archaeological findings.

The mechanism by which starch granules are incorporated into plaque and calculus remains largely unknown, and few studies have directly investigated potential mechanisms. We know that a proportion of the starch granules entering the mouth can become trapped in the plaque/calculus, and can be recovered from archaeological samples of considerable age (Buckley et al., 2014; Henry et

al., 2014; Wu et al., 2021). Studies have also shown that not all starch granules come from a dietary source. Other pathways include cross-contamination from plant interactions in soil, such as palm phytoliths adhering to the skin of sweet potatoes (Tromp & Dudgeon, 2015), or accidental ingestion not related to food consumption (Radini et al., 2017, 2019).

When starch granules enter the mouth, whether through ingestion of food or accidental intake, they immediately encounter multiple obstacles. It is likely that the bulk of starch granules are swallowed along with the food, and are only briefly present in the oral cavity. Other granules that are broken off during mastication may be retained in the dentition through attachment to tooth surfaces (including plaque and dental calculus) and mucous membranes (Dodds & Edgar, 1988; S. Kashket et al., 1991). Bacteria also have the ability to adhere to starch granules (Topping et al., 2003), which would allow starches to attach to bacterial communities within the biofilm. These granules are then susceptible to mechanical removal by the tongue, salivary clearance, and hydrolysis by α-amylase (S. Kashket et al., 1996). The susceptibility of granules to hydrolysis depends on the crystallinity and size of the starch granule, as well as the mode of processing. Smaller and pre-processed (e.g., cooked) starch granules are more susceptible to enzymatic degradation, while dehydrated starches will have a reduced susceptibility (Björck et al., 1984; Franco et al., 1992; Haslam, 2004; Henry et al., 2009; Lingstrom et al., 1994). Cracks on the surface of the dental calculus, as well as unmineralised islands and channels may also be able to contain starch granules (Charlier et al., 2010; R. C. Power et al., 2014; Tan, Gillam, et al., 2004). Starch granules that are trapped in these pockets are (at least to some extent) protected from aforementioned clearance mechanisms, especially once a new layer of plaque has covered the surface of the plaque/calculus. The size bias against large granules (>20 μm) from both wheat and potato (Table 4.4) may give further credence to this incorporation pathway, as the smaller starch granules have an advantage over larger granules, and can be stored in larger quantities. This was also suggested by Power and colleagues (2014), who observed clusters of starches within dental calculus, rather than an even distribution across the sur-

face of the dental calculus. Granules trapped in plaque/calculus may still be susceptible to hydrolysis, as α-amylase has the ability to bind to both tooth enamel and bacteria within a biofilm and retain a portion of its hydrolytic activity (Nikitkova et al., 2013; Scannapieco et al., 1993; Tan, Mordan, et al., 2004; Tan, Gillam, et al., 2004). After the death of an individual, starches within dental calculus are susceptible to further degradation by post-depositional processes, depending on burial environment (pH, temperature, moisture content, microorganisms) (Franco et al., 1992; García-Granero, 2020; Haslam, 2004; Henry et al., 2009). Future study should explore how burial affects the recovery of starch from the biofilm model.

The absence of α-amylase in the model is a limitation of this study, as the total granule counts were not subject to hydrolysis. This would likely have reduced and affected the size ratios, as smaller starches may be more susceptible to hydrolysis (Franco et al., 1992; Haslam, 2004). The absence may also affect biofilm growth due to the lack of amylase-bacterium interactions (Nikitkova et al., 2013). Conversely, the model may benefit from the absence of α-amylase, because it can allow us to directly explore its effect on starch counts in future experiments, where α-amylase can be added to the model in concentrations similar to those found in the oral cavity (Scannapieco et al., 1993). We are able to show how absolute counts in the treatments cause a difference in incorporation. However, this was merely a side-effect of the difference in the number of granules in potato and wheat solutions of the same concentration (w/v). Further research should test multiple differing concentrations of the same starch type. The use of EDTA may also have affected counts. While previous studies have shown negligible morphological changes caused by exposure to EDTA (Le Moyne & Crowther, 2021; Modi et al., 2020; Tromp et al., 2017), these studies have not considered changes to separate size categories within starch types, and whether shifts in size ratios occur due to exposure to the pre-treatment chemicals. The total number of granules on a slide often exceeded a number that was feasible to count in a reasonable time period, so we calculated the total counts by extrapolating from three slide transects. Thus, we reasonably assume

that the three transects are a good representation of the entire slide, and that the distribution of all granules on the slide is relatively homogeneous.
Finally, we only used native starches in the experimental procedure and the results will likely differ for processed starches (García-Granero, 2020). Based on the comparatively low counts obtained by Leonard and colleagues (2015, Supplement 2), processing and amylase may have a substantial effect on starch granule retention in the oral cavity.

While we are unable to sufficiently address the mechanism(s) of starch incorporation with the data obtained in this study, the dental calculus model presented here is uniquely suited to explore these questions and may improve interpretations of dietary practices in past populations. Further analyses using this model can address the call for more baseline testing of biases associated with dietary research conducted on dental calculus (Le Moyne & Crowther, 2021). Our high-throughput experimental setup allows us a higher degree of control over the factors that influence starch incorporation and retention, such as dietary intake, differential survivability of starches, and inter- and intra-individual variation in plaque accumulation and mineralisation. The latter is especially difficult to control *in vivo* as it is influenced by numerous factors including genetics, diet, salivary flow, and tooth position and morphology (Fagernäs et al., 2021; Haffajee et al., 2009; Jepsen et al., 2011; Proctor et al., 2018; Simón-Soro et al., 2013), as well as evolutionary differences (Fellows Yates et al., 2021). The set of limitations for our model differ from *in vivo* methods and, as such, we expect our model to complement the results and interpretations of existing and new *in vivo* studies. It can also facilitate training of students and researchers on methods of dental calculus analysis, such as starch and phytolith extraction and identification, where it can replace the use of finite archaeological resources.

4.5 Conclusion

This preliminary study shows that a very small proportion of the input starch granules are retained in a dental calculus model. This and previous studies have shown that calculus has a low capacity for retention of starch granules, an effect that is compounded by diagenetic effects in archaeological remains, resulting in low overall counts of extracted granules. The proportion of starches consumed will in many cases be reflected in the quantity of starches extracted from the dental calculus—i.e., the more starch granules entering the oral cavity, the more will be recovered from extraction—at least in modern calculus samples unaffected by diagenesis and hydrolysis. Whether or not this also applies to archaeological samples remains to be tested. Additionally, we have shown that the size of granules will influence the likelihood of incorporation, as large (>20 μm) starches have a decreased incorporation rate, medium (10–20 μm) starches an increased rate, and small (<10 μm) granules remained somewhat constant. The size of calculus deposit also seems to influence the capacity of granule incorporation; as the size of the deposit increases, so does the absolute count of incorporated granules.

While we have shown multiple factors that influence the likelihood of incorporation, the process still appears to be somewhat stochastic. Further research is needed to make sense of the contributing factors, and to explore the mechanisms of intra-oral starch incorporation and retention in dental calculus. The oral biofilm model described in this study provides a method to explore the incorporation and extraction of dietary compounds from dental calculus in a controlled laboratory setting. We do not expect our model to replace *in vivo* methods; instead, it can provide a complementary means to address the limitations of *in vivo* studies, and unearth the potential biases associated with dietary research conducted on archaeological dental calculus.

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5 | Article 3

Multiproxy analysis exploring patterns of diet and disease in dental calculus and skeletal remains from a 19th century Dutch population

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5.1 Introduction

Dental calculus has proven to be an excellent source of a wide variety of information about our past. The increased accessibility and advancement of methods in aDNA, paleoproteomics, and mass spectrometry, has expanded our ability to identify biomarkers of diet and disease on an increasingly large scale (Gismondi et al., 2020; Velsko et al., 2017; Warinner et al., 2014).

One such collection of biomarkers is alkaloids, a plant-derived group of compounds. Many alkaloids have important medicinal and psychoactive effects in humans, and their direct detection, or detection of their metabolites, is of great interest to archaeologists. Previous studies have successfully recovered alkaloids in archaeological contexts, including ceramics (Smith et al., 2018), pipes (Rafferty et al., 2012), human hair (Echeverría & Niemeyer, 2013; Ogalde et al., 2009), and even dental calculus employing both targeted (Eerkens et al., 2018) and untargeted approaches (Buckley et al., 2014; Gismondi et al., 2020). Especially nicotine, the principal alkaloid in tobacco leaves, has been widely studied in the archaeological record due to its apparent stability and ability to survive over long periods of time (Eerkens et al., 2018; Rafferty et al., 2012; Tushingham et al., 2013).

Alkaloids may enter the oral cavity via two pathways: (1) direct incorporation through ingestion of alkaloid-containing plants, whether deliberate or accidental; and (2) passive diffusion as alkaloids and other compounds are transferred from plasma to saliva, and then gradually secreted into the oral cavity through the salivary glands in the hours-to-days following ingestion (Cone & Huestis, 2007). The second pathway allows the identification of parent compounds that do not enter the mouth (e.g. injection), as long as they, or their metabolites, are excreted through the saliva, thus eventually entering the oral cavity.

Many of the components involved in the formation and growth of dental calculus originate from oral fluid. Proteins, bacteria, salts and other compounds are transferred from saliva to biofilms on the tooth surface (Jin & Yip, 2002;

White, 1997). This may also allow various alkaloids of dietary and medicinal origin to become incorporated in dental plaque. Dental plaque undergoes frequent mineralisation events, ultimately causing the entrapped alkaloids and their metabolites to become preserved within the dental calculus. Barring intentional or accidental removal of the calculus during life, burial, excavation, and post-excavation cleaning, the alkaloids can then be detected by various methods to show a record of consumption during life. Because drugs may be transferred from plasma to saliva, there is often a close correlation between drugs detected in oral fluid and blood, though there are differences in detected concentrations (Cone & Huestis, 2007; Milman et al., 2011; Wille et al., 2009). This was also shown to be true for dental calculus and blood (Sørensen et al., 2021), making dental calculus a potentially useful substance for detecting ancient alkaloids and other dietary compounds.

In this study we use a ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method that was developed in a previous study on dental calculus from cadavers and validated by comparing the results to compounds detected in the blood of the same individuals (Sørensen et al., 2021). All compounds that were detected in the blood were also detected in dental calculus, with additional compounds present in dental calculus that were not present in blood, suggesting that dental calculus represents a comprehensive history of consumption over a long period of time (Sørensen et al., 2021). We were able to detect both parent compounds and metabolites, including caffeine, nicotine, theophylline, and cotinine, in the dental calculus of individuals from a 19th century Dutch population from Middenbeemster. By detecting these compounds we are able to show the consumption of tea and coffee and smoking of tobacco on an individual scale, which is also confirmed by historic documentation and identification of pipe notches in the dentition.

5.2 Materials

The sample consists of 41 individuals from Middenbeemster, a 19th century rural Dutch site. The village of Middenbeemster and the surrounding Beemsterpolder was established in the beginning of the 17th century, when the Beemster lake was drained to create more farmland, mainly for the cultivation of cole seeds (de Vries 1978). In 1615, a decision was made to build a church, and construction started in 1618 (Hakvoort 2013). The excavated cemetery is associated with the Keyserkerk church, where the inhabitants of the Middenbeemster village and the surrounding Beemsterpolder were buried between AD 1615 and 1866 (Lemmers et al., 2013). Archival documents are available for those buried between AD 1829 and 1866, when the majority of individuals were interred. The main occupation of the inhabitants was dairy farming, consisting largely of manual labour prior to the industrial revolution (Aten et al., 2012; Palmer et al., 2016).

For our sample, we preferentially selected males from the middle adult age category (35-49 years) to minimise the effect of confounding cultural and biological factors. Previous research on Middenbeemster has shown a gendered division of labour (Palmer et al., 2016), and there are biological differences in dental calculus formation and drug metabolism that are related to age and sex (Huang et al., 2023; Uno et al., 2017; White, 1997). The sample consists of 27 males, 11 probable males, 2 probable females, and 1 female (Figure 5.1). We selected males due to a higher occurrence of pipe notches and dental calculus deposits than females (unpublished observation).

5.3 Methods

5.3.1 Skeletal analysis

Demographic and pathological analyses were conducted in the Laboratory for Human Osteoarchaeology at Leiden University. Sex was estimated using cra-

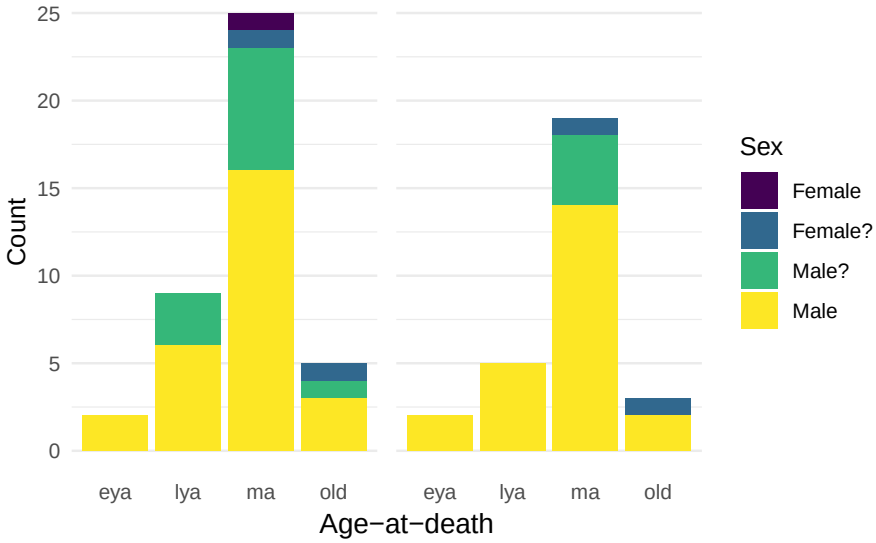

Figure 5.1 – Overview of sample demography. Left plot is the first batch and right plot is the replication batch with 29 of the individuals from the first batch. eya = early young adult (18-24 years); lya = late young adult (25-34 years); ma = middle adult (35-49 years); old = old adult (50+ years). Male? = probable male; Female? = probable female.

nial and pelvic morphological traits (Buikstra & Ubelaker, 1994). Age-at-death was estimated using dental wear, auricular and pubic surface appearance, cranial suture closure, and epiphyseal fusion (Brooks & Suchey, 1990; Buckberry & Chamberlain, 2002; Buikstra & Ubelaker, 1994; Lovejoy et al., 1985; Meindl & Lovejoy, 1985), and divided into the following categories: early young adult (18-24 years), late young adult (25-34 years), middle adult (35-49 years), old adult (50+ years). Preservation was visually scored on a four-stage scale (excellent, good, fair, poor) based on the surface condition of the bones and the extent of taphonomic degradation.

5.3.1.1 Paleopathology

Pathological conditions and lesions that occur frequently in the population were included in the analysis. Data were dichotomised to presence/absence to allow for statistical analysis. Osteoarthritis was considered present in cases where eburnation was visible on one or more joint surfaces. Vertebral osteophytosis is identified by marginal lipping and/or osteophyte formation on the margin of the superior and inferior surfaces of the vertebral body. Cribra orbitalia was diagnosed based on the presence of pitting on the superior surface of the orbit. No distinction was made between active or healing lesions. Degenerative disc disease, or spondylosis, is identified as a large diffuse depression of the superior and/or inferior surfaces of the vertebral body (Rogers, 2000). Schmorl's nodes are identified as any cortical depressions on the surface of the vertebral body. Data on chronic maxillary sinusitis from Casna et al. (2021) were included in this study to assess the relationship between upper respiratory diseases with environmental factors (i.e. tobacco smoke, caffeine consumption). Lesions associated with chronic maxillary sinusitis as defined by Boocock et al. (1995) were recorded for each individual and classified as "pitting", "spicule-type bone formation", "remodeled spicules", or "white pitted bone". chronic maxillary sinusitis was scored as absent when the sinus presented smooth surfaces with little or no associated pitting.

5.3.1.2 Dental pathology

Caries ratios were calculated by dividing the number of lesions by the number of teeth scored, resulting in a single caries ratio per individual. If the surface where the lesion originated is not visible, i.e. if the lesion covered multiple surfaces, this was scored as "crown". Calculus indices were calculated according to Greene and colleagues (2005). Calculus was scored with a four-stage scoring system (0-3) to score absent, slight, moderate, and heavy calculus deposits (Brothwell, 1981) on the lingual, buccal (and labial), and interproximal surfaces of each tooth. Only one score was used for the combined interproximal surfaces, resulting in three scores per tooth (when surfaces are intact), and four calculus indices per individual; upper anterior, upper posterior, lower anterior, lower posterior. Each index was calculated by dividing the sum of calculus scores for each surface by the total number of surfaces scored in each quadrant. If a tooth could not be scored on all three surfaces, the tooth was not included (Greene et al., 2005). Periodontitis was scored on a visual four-stage (0-3) scoring system according to distance from cemento-enamel junction of each tooth to alveolar bone (Maat & Mastwijk, 2005).

5.3.2 Calculus sampling

Where possible, we used material that had already been sampled for a previous study to prevent unnecessary repeated sampling of individuals. Calculus from the previous study was sampled in a dedicated ancient DNA laboratory at the Laboratories of Molecular Anthropology and Microbiome Research in Norman, Oklahoma, U.S.A, using established ancient DNA protocols. More details on the methods can be found in the published articles (Ziesemer et al., 2015, 2018). Of the 41 individuals that were originally included in our sample, 29 were replicated in a separate analysis only using calculus from the previous study.
New dental calculus samples were taken under sterile conditions in a positive pressure laminar flow hood in a dedicated dental calculus lab at Leiden University. The surface of the tooth was lightly brushed with a sterile, disposable

toothbrush to get rid of surface contaminants. A sterile dental curette was then used to scrape calculus from the tooth onto weighing paper, which was transferred to 1.5 ml Eppendorf tubes. All calculus samples were sent to the Department of Forensic Medicine at Aarhus University for ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis.

5.3.3 UHPLC-MS/MS

The list of targeted compounds included both naturally occurring compounds known to have been used in the past, as well as synthetic modern drugs that did not exist at the time (e.g. Fentanyl, MDMA, Amphetamine). These were part of the toxicology screening for the original method (Sørensen et al., 2021), developed on cadavers. In our study they serve as an authentication step, as their presence in archaeological samples could only be the result of contamination.

Briefly, samples of dental calculus were washed three times each with one mL of methanol (MeOH), to remove surface contaminants. The wash solutions were collected separately. The solvent was evaporated and the residues were dissolved in 50 µL 30% MeOH. The washed calculus was homogenized in presence of 0.5 M citric acid using a lysing tube with stainless steel beads. Following one hour of incubation the dissolution extract was cleaned by weak and strong cation-exchange. After evaporation of the elution solvent the residue was dissolved in 50 µL 30% MeOH. The final extracts obtained from washing and dissolution of the dental calculus were analysed by UHPLC-MS/MS using a reversed-phase biphenyl column for chromatography. To obtain quantitative results, isotope dilution was applied. For more details about the method and validation, see the original study by Sørensen and colleagues (2021).

5.3.4 Statistical analysis

All compounds and pathological conditions/lesions were converted to a presence/absence score. Pearson product-moment correlation was applied to the

dichotomised pathological lesions (point-biserial correlation), compound concentrations, calculus indices, and caries ratios to explore relationships paired continuous-continuous variables and paired continuous-binary variables. Compound concentrations were then dichotomised to presence/absence, and the caries ratio and calculus index for each individual were converted to an ordinal score from 0 to 4 by using quartiles. Polychoric correlation was applied to the paired dichotomous variables and dichotomous-ordinal variables.

All statistical analysis was conducted in R version 4.3.3 (2024-02-29), Angel Food Cake, (R Core Team, 2020). Data wrangling was conducted with the **tidyverse** (Wickham et al., 2019) and visualisations were created using **ggplot2** (Wickham, 2016). Polychoric correlations were calculated with the **psych** package (Revelle, 2022).

5.4 Results

Multiple compounds were detected in the dental calculus samples. Compounds detected at a lower concentration than the lower limit of quantitation (LLOQ) were considered not present. Not all the compounds detected in the first batch could be replicated in the second batch (Table 5.1). For a full list of targeted compounds, see Supplementary Material.

Table 5.1 – Target compound including whether it was detected (TRUE) or not (FALSE) in each batch, as well as the lower limit of quantitation (LLOQ) in ng. CBD = cannabidiol; CBN = cannabinol; THC = tetrahydrocannabinol; THCA-A = tetrahydrocannabinolic acid A; THCVA = tetrahydrocannabivarin acid.

Compound	Batch 1	Batch 2	LLOQ
CBD	TRUE	FALSE	0.050
CBN	TRUE	FALSE	0.050
Caffeine	TRUE	TRUE	0.050
Cocaine	TRUE	FALSE	0.025
Cotinine	TRUE	TRUE	0.050

Table 5.1 – Target compound including whether it was detected (TRUE) or not (FALSE) in each batch, as well as the lower limit of quantitation (LLOQ) in ng. CBD = cannabidiol; CBN = cannabinol; THC = tetrahydrocannabinol; THCA-A = tetrahydrocannabinolic acid A; THCVA = tetrahydrocannabivarin acid.

Compound	Batch 1	Batch 2	LLOQ
Nicotine	TRUE	TRUE	0.100
Salicylic acid	TRUE	TRUE	0.500
THC	TRUE	FALSE	0.100
THCA-A	TRUE	FALSE	0.025
THCVA	TRUE	FALSE	0.010
Theophylline	TRUE	TRUE	0.010

The pattern we expect to see in authentic compounds representing compounds trapped within the dental calculus, is a reduction in the quantity from wash 1 to wash 3 as potential surface contaminants are washed off, and then a spike in the final extraction when entrapped compounds are released and detected.

Most plots show a large increase in extracted mass of a compound between the calculus wash extracts (wash 1-3) and the dissolved calculus (calc). Most samples containing theophylline and caffeine had the largest quantity of the compound extracted from the first wash, then decreasing in washes 2 and 3. There is an increase between wash 3 and the dissolved calculus in all samples. The patterns are consistent across batches 1 and 2. Nicotine and cotinine have the same relative quantities in the samples, i.e., the sample with the highest extracted quantity of nicotine also had the highest extracted quantity of cotinine (Figure 5.2).

To see if preservation of the skeletal remains had any effect on the detection of compounds, we compare extracted quantities of compounds to the various levels of skeletal preservation. Our results from batch 2 suggest that detection of a compound may be linked to the preservation of the skeleton, with better

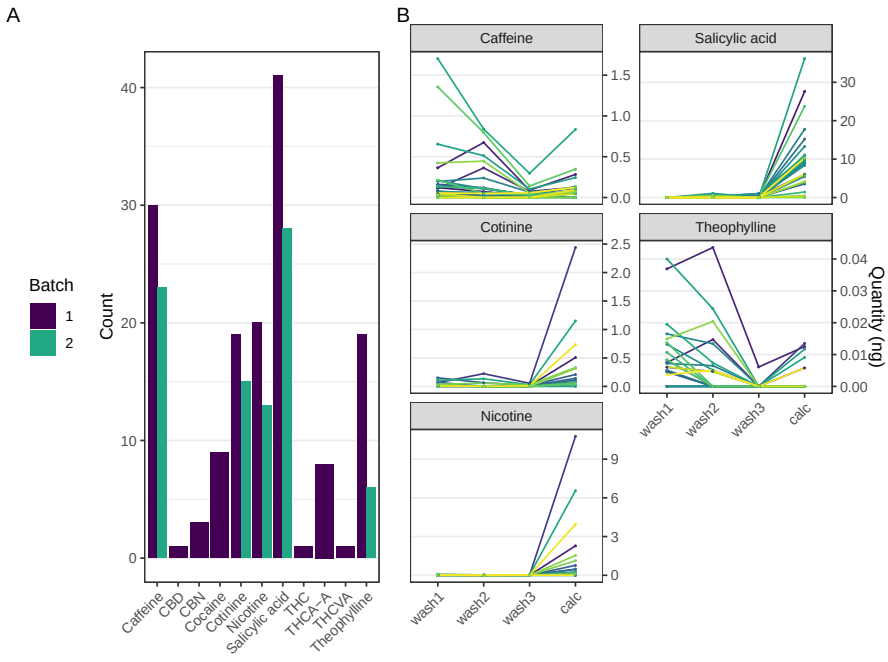

Figure 5.2 – (A) Number of samples in which each compound was detected in the first and second batch. (B) Quantity (ng) of each compound extracted from each sample in batch 2. The plot displays the extracted quantity across the three washes and final calculus extraction (calc). Each coloured line represents a different calculus sample. CBD = cannabidiol; CBN = cannabinol; THC = tetrahydrocannabinol; THCA-A = tetrahydrocannabinolic acid A; THCVA = tetrahydrocannabivarin acid.

preservation leading to increased extraction quantity (Figure 5.3A). We also find a weak positive correlation between the weight of the calculus sample and the quantity of compound extracted from the calculus (Figure 5.3B).

The presence of pipe notch(es) in an individual and concurrent detection of nicotine and/or cotinine is used as a crude indicator of the accuracy of the method. Only males were used in accuracy calculations, as pipe notches are ubiquitous in males, but not in females. In batch 2, the method was able to detect some form of tobacco in 14 of 25 individuals with a pipe notch (56.0%). When also considering correct the absence of a tobacco alkaloid together with the absence of a pipe notch, the accuracy of the method is 59.3%. Accuracy in the old adult age category is 100.0%, but with only 2 individuals.

One individual—an old adult, probable female—was positive for both nicotine and cotinine, and had no signs of a pipe notch.

5.4.1 Correlations between detected alkaloids and diseases

For further statistical analyses, only the UHPLC-MS/MS results from batch 2 were used, as batch 1 had multiple compounds that were not detected in batch 2 and may have been contaminated.

Table 5.2 – Pearson correlation (*r*) on dichotomous skeletal lesions and compound concentrations (ng/mg) from the second batch. Correlations between pairs of dichotomous variables are removed due to incompatibility with a Pearson correlation. Moderate and strong correlations in **bold**. OA = osteoarthritis; VOP = vertebral osteophytosis; SN = Schmorl's nodes; DDD = degenerative disc disease; CO = cribra orbitalia; CMS = chronic maxillary sinusitis; SA = salicylic acid; PN = pipe notches.

	Caries	Nicotine	SA	Calculus	PN	Theophylline	Caffeine	Cotinine
OA	-0.12	-0.07	0.21	0.07	0.14	0.28	0	-0.07
VOP	-0.09	-0.16	0.34	0.06	0.25	-0.06	0.01	-0.13
SN	-0.24	0.16	0.09	0.09	0.17	0.24	0.16	0.09
DDD	0	0	0.19	-0.39	-0.08	0.31	0.06	-0.01

Table 5.2 – Pearson correlation (*r*) on dichotomous skeletal lesions and compound concentrations (ng/mg) from the second batch. Correlations between pairs of dichotomous variables are removed due to incompatibility with a Pearson correlation. Moderate and strong correlations in **bold**. OA = osteoarthritis; VOP = vertebral osteophytosis; SN = Schmorl's nodes; DDD = degenerative disc disease; CO = cribra orbitalia; CMS = chronic maxillary sinusitis; SA = salicylic acid; PN = pipe notches.

	Caries	Nicotine	SA	Calculus	PN	Theophylline	Caffeine	Cotinine
CO	0.06	-0.05	0.2	0.14	-0.2	-0.11	0.19	-0.06
CMS	-0.19	0.28	0	-0.27	0.03	0.19	0.36	0.22
Caries		-0.2	-0.36	-0.15	-0.17	-0.21	0	-0.22
Nicotine			-0.21	0.01	-0.01	**0.43**	0.14	**0.98**
SA				0.14	0.37	0.04	0.17	-0.17
Calculus					0.13	-0.15	-0.13	0.03
PN						-0.16	0.18	-0.01
Theophylline							**0.51**	0.36
Caffeine								0.08

Point-biserial correlation was conducted on paired continuous and dichotomous variables, to see if any relationships exist between extracted concentrations and other variables. The strongest point-biserial (Pearson) correlation correlations were a near-perfect positive correlation between cotinine and nicotine (0.98), and moderate correlations between theophylline and nicotine (0.43), caffeine and theophylline (0.51) (Table 5.2).

Polychoric correlation was conducted on the dichotomised compounds and pathological conditions, as well as the discretised dental diseases. Salicylic acid was removed due to its ubiquitous presence in the sample, and is likely to cause spurious correlations. Strong correlations were found between cotinine and nicotine (0.85). Moderate correlations were found between OA and DDD (0.47), VOP and periodontitis (0.49), SN and cotinine (0.56), DDD and calculus (-0.42), CMS and caffeine (0.53), caries and periodontitis (0.52), periodontitis

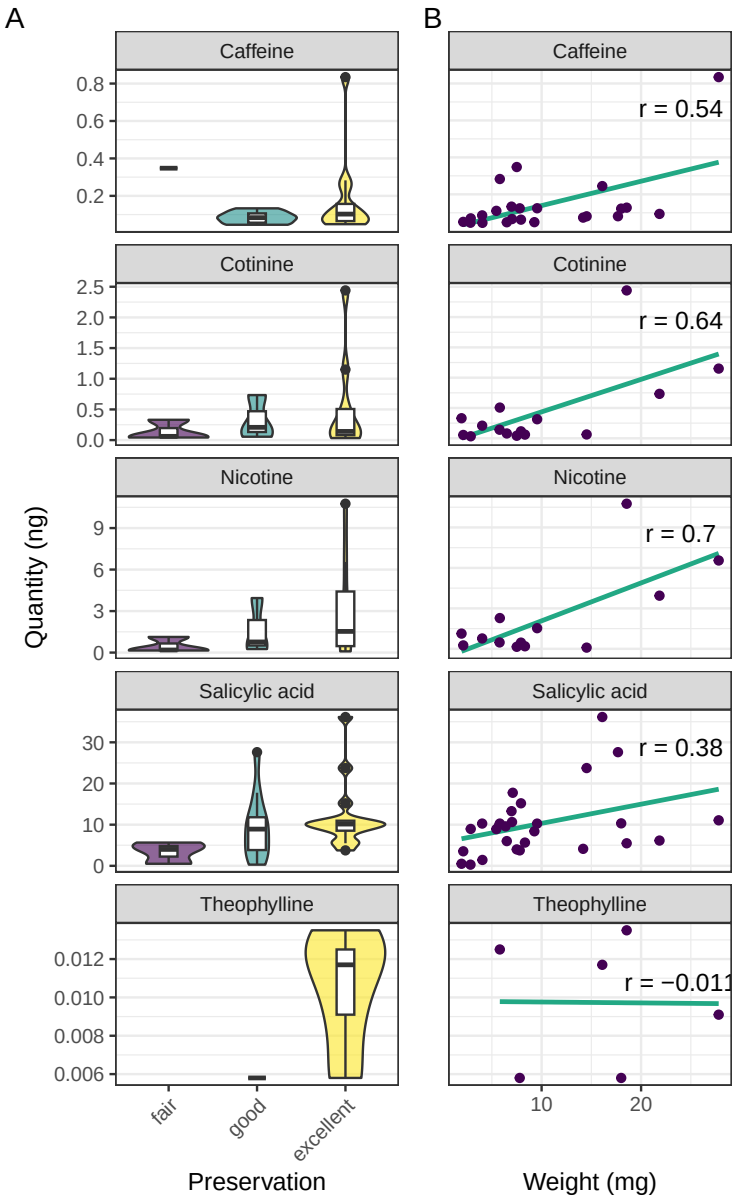

Figure 5.3 – (A) Violin plot with overlaid box plots depicting the distribution of extracted quantities of each compound from batch 2 separated by state of preservation of the skeleton. (B) Extracted quantity (ng) of compound plotted against weights of the calculus samples from batch 2. r = Pearson correlation coefficient.

and VOP (0.49), periodontitis and age-at-death (0.41), nicotine and SN (0.53), calculus and DDD (-0.42), age-at-death and theophylline (-0.45), theophylline and age-at-death (-0.45), caffeine and periodontitis (0.49), cotinine and CMS (0.43). Remaining correlations were weak or absent (Figure 5.4). Correlations with age will be depressed because age was largely controlled for in the sample selection.

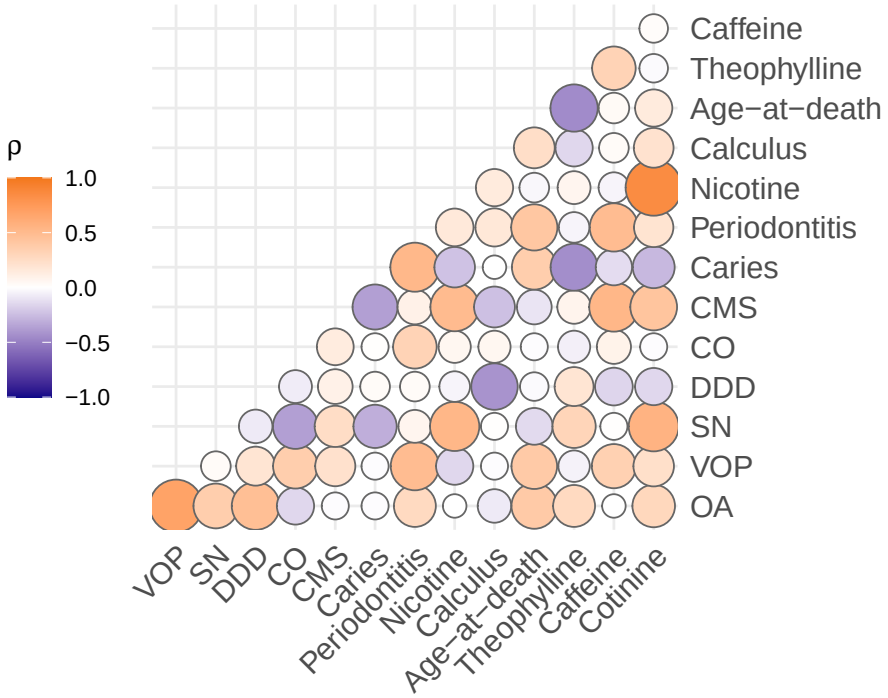

Figure 5.4 – Plot of the polychoric correlations (*rho*). Larger circles and increased opacity indicates a stronger correlation coefficient. OA = osteoarthritis; VOP = vertebral osteophytosis; SN = Schmorl's nodes; DDD = degenerative disc disease; CO = cribra orbitalia; CMS = chronic maxillary sinusitis; SA = salicylic acid.

5.5 Discussion

In this study we were able to extract and identify multiple alkaloids and salicylic acid from the dental calculus of individuals from Middenbeemster, a 19th century Dutch archaeological site. We applied ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) using a method that was validated by co-occurrence of drugs and metabolites in dental calculus and blood (Sørensen et al., 2021). Here we have shown that the method can also be successfully applied to archaeological dental calculus. We extend findings from previous studies on alkaloids in archaeological samples by detecting multiple different alkaloids in dental calculus, including nicotine, cotinine, caffeine, theophylline, and salicylic acid. The detection of these compounds was solidified in a replication analysis on different samples from the same individuals. Cocaine and multiple cannabinoids were also detected during the first analysis, but were not replicated. We contextualize these findings within the historical and archaeological evidence for consumption of these drugs and dietary compounds.

Nicotine and its principal/main metabolite, cotinine, were strongly positively correlated, both in concentration and presence/absence in individuals (Table 5.2 and Figure 5.4). The detection of nicotine and cotinine is not surprising, as pipe-smoking in the Beemsterpolder is well-documented in the literature (Aten et al., 2012; Bouman, 2017), and visible on the skeletal remains as pipe notches (Lemmers et al., 2013). There is also documented medicinal use of nicotine in the Beemsterpolder, where a tobacco-smoke enema was used for headaches, respiratory problems, colds, and drowsiness from around 1780 to 1830 (Aten et al., 2012). In our sample, we also detected nicotine and cotinine (replicated) in an old adult, probable female individual. In this particular case it is unlikely that the compounds entered the dental calculus through pipe-smoking, as the individual had no visible pipe notches; more likely the tobacco entered through an alternate mode of consumption, secondhand smoke, or the aforementioned tobacco-smoke enema.

Theophylline and caffeine were positively correlated in our samples, though to a lesser extent than nicotine and cotinine, so we are unable to determine if they originated from the same source (Table 5.2 and Figure 5.4). Caffeine and theophylline have very similar chemical structures, so we expect they would experience similar rates of incorporation and degradation, allowing us to interpret the ratio and correlations between the compounds. Caffeine is present in coffee, tea, and cocoa beans, with concentrations slightly higher in coffee (Bispo et al., 2002; Chin et al., 2008; Srdjenovic et al., 2008; Stavric et al., 1988). Theophylline is present in both coffee beans and tea leaves, but in negligible quantities (Stavric et al., 1988). It is also a primary metabolite of caffeine produced by the liver. Given the low correlation, there are likely multiple sources of caffeine and theophylline in the population, with tea and coffee being the most obvious.

Tea consumption had become widespread in the Netherlands by 1820, reaching all parts of society (Nierstrasz, 2015, p. 91). Historically, we also know that both tea and coffee were consumed in the Beemsterpolder during the 19th century. 'Theegasten' (teatime) was a special occasion occurring from 15.00-20.00 hours, where tea was served along with the evening bread (Schuijtemaker, 2011). Many households also owned at least one coffee pot and tea pot (Bouman, 2017). Distinguishing between tea, coffee, and chocolate may be possible by also including theobromine and comparing ratios of the compounds, as theobromine is present in higher quantities in chocolate compared to caffeine and theophylline (Alañón et al., 2016; Bispo et al., 2002; Stavric et al., 1988). However, In addition to oral factors affecting alkaloid uptake in dental calculus, there is some indication that theobromine does not preserve well in the archaeological record (Velsko et al., 2017), and frequent consumption of all three items would be difficult to parse. Additionally, we do not understand well enough the effect of the burial on these specific compounds, and the original concentration of the compounds in plants can be quite variable (King et al., 2017).

Salicylic acid was found in all but one individual in our sample. It can be extracted from the bark of willow trees, *Salix alba*, and has long been used for its

pain-relieving properties (Bruinsma, 1872, p. 119). It is also present in many plant-based foods (Duthie & Wood, 2011; Malakar et al., 2017), including potatoes, which were a staple of the Beemsterpolder diet (Aten et al., 2012). The extracted quantity from our samples decreased over the three washes, followed by a sharp increase in the final calculus extraction, which is what we would expect to see if the salicylic acid was incorporated during life (Figure 5.2). It is important to note that, especially with salicylic acid, there is a possibility for the compound to enter the calculus through contact with the surrounding soil. Salicylic acid is a very mobile organic acid (Badri & Vivanco, 2009; Chen et al., 2001) and the ubiqutous presence in our samples may be explained by the compound leching into the dental calculus from the burial environment. We can therefore not confidently rule out environmental contamination without analysing samples from the surrounding soil.

Cannabinoids—specifically THC, THCA-A, THCVA, CBD, CBN—were found in the first batch, but none were replicated in the second batch. Medicinal use of cannabinoids has been well-established in Europe since Medieval-times, and it was also grown in the Netherlands (Bruinsma, 1872). Administration was most common in the form of concoctions containing various portions of the cannabis plant for ingestion; not until the late 19th century did it become recommended to smoke it for more immediate effects (Clarke, 2013). Dutch medicinal preparations were used to treat a variety of ailments and symptoms, including pain, inflammation, various stomach ailments, gout, and joint pains (Clarke, 2013). Because cannabinoids have an affinity for protein-binding, they are less likely to diffuse from serum to saliva (Cone & Huestis, 2007). This may make them difficult to detect in dental calculus unless the cannibinoids were consumed orally; even then, the overall instability of some cannabinoids could also limit detection (Lindholst, 2010; Sørensen & Hasselstrøm, 2018). Given the lack of replication, we cannot with security confirm that cannabis was used by the Beemster population.

Despite many of our sampled individuals having lived during the height of the

opium era in the Netherlands (Macht, 1915), none of the targeted opioids (morphine, codeine, thebaine, papaverine, norcodeine, noscapine) were detected. The absence of opioids could be a result of the people ascribing more to the "traditional" rather than "scientific" medicine, although laudanum and another opium containing concoction was part of the "traditional" medicine in the Netherlands (Leuw & Marshall, 1994), including Middenbeemster (Aten et al., 2012). It was also generally considered a drug of the upper class (Scheltema, 1907), and may have been more common in urban centers. The absence could also be attributed to postmortem degradation. It has been shown that, while morphine is abundant in opium, it degrades rapidly. Thebaine and papaverine are more resistant to various ageing processes (Chovanec et al., 2012), however, these were also absent from our samples.

The only strictly modern compound (at least in a European context) detected in the sample was cocaine, which was detected in the first batch of samples. Our sample is derived from an early–mid 19th century population, and cocaine was isolated in 1860 by Albert Niemann, and entered popular medical practice in 1884. Coca arrived in Europe as early as 1771, but as botanical specimens rather than for consumption, and there were also issues importing enough viable specimens of coca for cocaine extraction (Abduca, 2019, p. 108; Mortimer, 1901, p. 179). This would have been the first case of coca-leaf-consumption in Europe; however, we were unable to replicate any of the cocaine results in the second batch. We suspect that the original detection of cocaine was a result of lab contamination during analysis.

We explored the relationship between detected compounds and various skeletal indicators, such as pathological and dental lesions, preservation, and pipe notches. We found some evidence to suggest that preservation of the skeleton influences the recovery of compounds from the dental calculus, with well-preserved skeletons potentially serving as a better target for sampling.
We found a positive correlation between CMS and nicotine, which may be indicative of the impact tobacco smoking had on the respiratory health of the

Beemster inhabitants. Tobacco smoke may play a significant role in diseases of the upper respiratory tract, including chronic maxillary sinusitis (Reh et al., 2012). Although the mechanisms by which smoking increases the risk of infections is not fully understood, solid evidence has been presented linking tobacco smoke to increased mucosal permeability and impairment of mucociliary clearance (Arcavi & Benowitz, 2004). Such changes, together with an altered immunologic response, are thought to predispose to the development of chronic maxillary sinusitis (Slavin et al., 2005).
We also observed a moderate positive correlation between chronic maxillary sinusitis and caffeine which contradicts previous research linking chronic coffee consumption with a positive effect on the respiratory system, suggesting a preventive association between caffeine intake and pneumonia (e.g. Alfaro et al., 2018; Kondo et al., 2021). However, while the lower respiratory tract seems to benefit from chronic coffee consumption, it is possible that elevated caffeine intake impacts mucosal moisture due to its dehydrating effect (Maughan & Griffin, 2003), thereby exposing individuals to greater risk of sinus infection.

The detection of nicotine in dental calculus has previously been presented by Eerkens and colleagues (2018) in two individuals from pre-contact California. They also targeted caffeine, cotinine, and theophylline in their samples, but were unable to detect any of them. It remains to be seen whether this is due to differences in methods used, or due to our samples being more recent. They also suggest that the choice of tooth for sampling may impact the detection of certain compounds, as the incorporation in dental calculus may depend on the mode of consumption. Tobacco smokers may have more nicotine present in calculus on incisors, whereas tobacco chewers may have more on molars (Eerkens et al., 2018). However, sampling may not be limited to mode of consumption. The presence of cotinine suggests that the excretion of a compound after being metabolised in the body is also a source of deposition, and that deposition of alkaloids in dental calculus can occur both on the way into the body, i.e. during consumption, and on the way out, i.e. disposal of waste products via saliva secretion into the mouth. Especially mucin-rich saliva from the sublingual and

submandibular glands preferentially binds toxins (Dodds et al., 2005), and since these glands are located closest to the lower incisors, they may be the most effective target for these studies. This has yet to be systematically tested in archaeological dental calculus. Because we homogenised samples from multiple teeth of an individual, we were unable to test the effect of oral biogeography. It is also possible that resident microflora within biofilms contribute to alkaloid breakdown and that the presence of caffeine and nicotine metabolites following direct ingestion can be explained by this pathway. However, the literature on biofilm biodegradation of alkaloids is limited, and *in vitro* studies have only found minimal contributions by certain oral bacteria in isolation (Cogo et al., 2008; Sun et al., 2016); it is possible that a larger role is played by oral bacteria within larger, more metabolically active communities, e.g. biofilms (Takahashi, 2015).

Targeting individuals with moderate-to-large calculus deposits likely biased our sample, as the presence of calculus may increase the risk of premature death (Yaussy & DeWitte, 2019). Additionally, periodontal disease (often linked to the presence of calculus) is a risk-factor for respiratory diseases, if periodontal and respiratory pathogens enter the bloodstream (Azarpazhooh & Leake, 2006; Scannapieco, 1999; Scannapieco & Ho, 2001). In our sample, the percentage of chronic maxillary sinusitis (37.0%) is lower than in another (more representative) male sample (44.1%) (Casna et al., 2021), and the caries percentage is similarly lower in our sample (17.6%) than a more representative sample (22.9%) (Lemmers et al., 2013).

We used the presence/absence of a pipe notch and concurrent detection of tobacco as a crude estimate of the accuracy of the method, which we found to be around 59.3%. This is a very rough estimate, as the presence of a pipe notch is likely not a perfect indicator of whether or not someone consumed tobacco. Dental calculus is also more transient than for example bone, as it can become dislodged during life, intentionally or unintentionally, eliminating all trace of the alkaloids consumed prior to its removal.

Following burial, compound stability over time will play a large role, as will mi-

crobial degradation of compounds by bacteria and fungi in soil (Liu et al., 2015), as well as the soil environment, such as temperature, pH, and oxygen availability (Lindholst, 2010; Mackie et al., 2017).

Due to this, quantitation of the detected compounds may have limited value in archaeological samples due to degradation, and will greatly affect our correlations related to concentration. The detected quantity of a compound will also depend on the quantity in dental calculus during life, which is largely controlled by the quantity consumed, how often the calculus was disrupted/removed, metabolic breakdown of the compound, and inter- and intra-individual factors related to stages of biofilm maturation (Lustmann et al., 1976; Velsko et al., 2019; Zijnge et al., 2010). In short, this means it is not really possible to equate the absence of a compound as evidence for the absence of consumption, which complicates the interpretation of our results. We have attempted to minimise errors occurring due to this limitation by including a relatively large sample of individuals and replicating our analysis. Although, given the relatively low detection rate seen in tobacco, this remains a major limitation, and will likely be compounded by increasing antiquity of the samples.

Future studies should explore how sampling from various types of teeth and their position in the mouth affects the probability of a compound becoming entrapped in dental calculus. This may also be related to properties within the oral cavity, as well as chemical properties of the compounds, which facilitate or reduce the incorporation-potential, and which incorporation pathways are more likely for a given compound.

We only targeted drugs that were included in the forensic toxicological screenings, and therefore only covered a limited number of the potential compounds that could be of interest for exploring past diets and medicinal treatments. The list of targeted compounds can be expanded as we discover more potential targets based on which specific compounds/metabolites are more likely to be incorporated and preserved in dental calculus.

There is an increasing interest in using oral fluid as a means of detecting alkaloids in living individuals due to the non-invasive nature of the testing com-

pared to blood and urine sampling (Cone, 1993; Valen et al., 2017). These *in vivo* studies are a valuable source of method validation and can help determine the feasibility of detecting certain alkaloids in oral fluid and, subsequently, dental calculus. Archaeologists, though, will likely be responsible for exploring dental-calculus-specific incorporation and retention of alkaloids, as well as their long-term preservation in the burial environment. Finally, following our experience with salicylic acid, we encourage all future studies to ensure that a control sample is taken from the soil, either from the soil surrounding the individual, or, ideally, directly from the skeletal remains. This should preferably happen before cleaning, but there will often be soil left over in cavities (e.g. nasal cavity, orbit, auditory meatus).

While a major limitation is the uncertainty surrounding whether or not a compound is actually absent, the power of the method lies in the ability to detect dietary and other compounds that were incorporated via multiple consumption pathways that are not detected by other methods. Taking tobacco consumption as an example; while pipe notches are a useful way to identify tobacco consumption, pipe smoking was not the only mode of tobacco consumption, with others including chewing, drinking, cigars, and snuff (Goodman, 1994, p. 67). Pipe-smoking was mainly practised by males (Eerkens et al., 2018; Lemmers et al., 2013), so methods like the one presented here are suitable for exploring tobacco consumption in an entire society, rather than a trivial subset of past populations. Combined with other methods, it can also give us a more complete picture of dietary patterns and medicinal/recreational plant-use in the past by capturing multiple possible incorporation pathways of dietary (and other) compounds.

5.6 Conclusion

This preliminary study outlines the benefits of using calculus to target a variety of compounds that could have been consumed as medicine or diet. This method allows us to directly address specific individuals, which can be espe-

cially useful in individuals that are not always well-documented in historic documentation, such as rural communities, children and women. We also show that there are many limitations that will need to be addressed going forward with this type of analysis, and stress the need for more systematic research on the consumption of alkaloid-containing items and their subsequent concentration and preservation in dental calculus, in addition to how mode of consumption may affect concentrations on different parts of the dentition. Another limitation of dental calculus as a medium is the inter- and intra-individual variability of its formation and the many factors that can influence incorporation and retention of molecules and particles; however, in the absence of hair and serum (quite uncommon in archaeology), dental calculus represents an impressive long-term reservoir of information regarding the consumption of various alkaloids, whether dietary, medicinal, recreational, or otherwise.

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6 | Discussion

Archaeological researchers are presented with a unique challenge. Because time eventually degrades everything, the archaeological record will always be incomplete. Barring the invention of time travel—and depending on your position on travelling back to a time before time travel is invented—we are limited in our ability to fill these gaps in our knowledge. Consider it a puzzle that needs to be put back together. The only problem is that some pieces are permanently missing, while the rest are mostly broken. Researchers will attempt to complete the puzzle by fixing the broken pieces with scientific analyses, and recreate the missing pieces based on what we can see from the broken pieces. To further complicate things, the methods we use to recreate the broken pieces may not be able to entirely accurately recreate the pieces, which results in pieces that look like they fit, but are actually different from the originals. Dental calculus is an example of a puzzle with many missing and broken pieces. Even if we analysed dental calculus from a living person, we would still not be able to completely recreate the entirety of that person's diet by only looking at the food debris within the dental calculus. For whatever reason, some of the things we eat will leave traces on our teeth, while some will not. Now add to that a few hundred or thousand years in the ground with physical and chemical processes that are constantly degrading the organic material, and the picture becomes even murkier. We can show something is there if we detect it. But what about the things we don't detect? Were they not there, or could we not detect them? If they weren't

there, why weren't they there? If the thing in question was consumed, but not entrapped in the dental calculus; why is this the case?

As shown in Chapter 1, dental calculus has become a very popular substance within archaeological research. One of its primary uses is to reconstruct the diet of past populations. It's not surprising why this is the case. It forms and grows inside our mouth over time, and it is in direct contact with everything we put in our mouth. However, there is limited systematic and fundamental research and experimentation being conducted within the fields that make use of archaeological dental calculus. There are of course exceptions (Fagernäs et al., 2021; Leonard et al., 2015; R. C. Power et al., 2015; Robert C. Power et al., 2021; Soto et al., 2019; Tromp et al., 2017; Velsko et al., 2019, 2023), but they have not addressed the full extent of dental calculus limitations (nor should they). This type of research should aim to validate aspects of our current analytical methods on synthetic materials or through detailed observation and documentation of dietary habits in living humans (or non-human primates), and critically evaluate the patterns of information we extract. Methods-validation has also been conducted on archaeological material (Fagernäs et al., 2021; Modi et al., 2020; Tromp et al., 2017), but these studies are limited by the fact that we have no way of knowing what the original diet looked like. At least not at the resolution necessary to really scrutinize the results of a method. All we have are pieces of information from the, likely incomplete, dietary remains that ended up in the calculus, and from contextual remains, such as animal bones, food residues, and plant remains, both macro- and microscopic. And even then we have no way of saying for certain whether the materials were included in the diet, or just there because our somewhat crucial requirement for oxygen means the oral cavity is not a closed system (Radini et al., 2017).

In this dissertation, I have mainly focused on the development, validation, and application of an oral biofilm model and its potential for informing archaeological research. I have shown that it was possible to develop a protocol for an oral biofilm model with a relatively simple setup, and use it to grow artificial dental

calculus, and that it can serve as a reasonable proxy to natural dental calculus [Chapter 3; Bartholdy, Velsko, et al. (2023)). I demonstrated how the oral biofilm model can answer questions and identify hidden biases related to using dental calculus for paleodietary reconstructions, specifically addressing the identification and quantification of starch granules. The results from this study showed that what goes in, doesn't necessarily come out. And the loss of information is not evenly distributed across the different types of starches, depending on size and morphology [Chapter 4; Bartholdy & Henry (2022)]. In Chapter 5 I present a study that goes beyond the model and looks at archaeological dental calculus. This is, after all, a dissertation in archaeology. We analysed dental calculus samples from a rural Dutch archaeological site in Middenbeemster, using ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-ESI-MS/MS). This allowed us to identify a number of residues from plants that may have been consumed for nutrition, medicine, recreation, or all of the above.

6.1 The dental calculus model

While the use of oral biofilm models in dental research is well-established, even long-term calcifying models to produce dental calculus, they never made it into archaeological research, at least not to the extent that the results or protocols of these models were published (that I could find). The oral biofilm model outlined in this dissertation is by no means the ultimate solution to save us from the limitations of archaeological dental calculus, but may provide a small step towards understanding them a little better, and hopefully promote further exploration through systematic fundamental research. The goal of developing a dental calculus model was to explore core aspects of how we use dental calculus in paleodietary research, with a relatively simple setup that is accessible to most labs in archaeological science. The idea is to take a step back and really scrutinise our current methods for interpreting diet from dental calculus. What the field has accomplished so far is undeniably impressive, but there are many things we still don't understand. Some of the things we don't understand are

on a very basic level, such as how plant microremains become trapped inside calculus, how much of what we consume ends up inside calculus, and to what extent our current methods are able to accurately extract that information.

The model we chose was a simple model using a shaking incubator and a 24 deepwell plate with the plastic lids as a substratum. The artificial saliva we used was based on the basal modified medium used by Sissons and colleagues (1991, 1994; 1997) to grow dental calculus. We also made use of their calcifying solution, calcium phosphate monofluorophosphate urea (CPMU) to speed up the mineralisation process (natural dental calculus can take weeks, even months, to form). To make sure the calculus we were growing in the lab was a good model for calculus grown naturally, we sequenced the DNA of our model calculus and compared it to samples from various sites inside the human mouth, including dental plaque and calculus. The bacterial composition of our model calculus samples had a strong oral signature, but was distinct from other natural oral samples, including modern dental plaque and calculus. The main difference between natural samples and model calculus was that the natural samples were more heterogeneous in composition, which is expected when comparing natural and lab-grown samples. Natural samples had a larger number and variety of microbes compared to the model calculus. This was reflected in the aerotolerance of dominant microbes in model calculus, which were largely anaerobes, while the most abundant microbes in natural samples were aerobes and facultative anaerobes. The natural samples also had a more diverse representation of bacteria from all stages of biofilm development, including early- middle-, and late-colonisers, while model calculus samples were predominantly late-colonisers (**Chapter 3**, Bartholdy, Velsko, et al. (2023)). Results from our metagenomic analysis were similar to a comparable *in vitro* biofilm model. In their study, the authors also used a 24-well plate with pooled saliva as inoculate. The growth medium was similar but also contained a sheep's-blood serum, and the samples were only grown for 24 hours (Edlund et al., 2018). As with our model, the comparison with natural oral samples showed a lower overall richness and diversity, and a distinct microbial profile (**Chapter 3**, Bartholdy, Velsko, et al. (2023)). Given

that our results are similar to a short-term biofilm model, we may be replacing the medium too often (every three days), and not allowing communities to establish more complex metabolic pathways that are normally present in mature biofilms. To resolve this and other issues, our protocol will benefit from further refinement. Using serum in the medium may help to establish thicker and more stable biofilms, and allow slow-growing organisms to become more established (Ammann et al., 2012). Filter-sterilising the heat-sensitive solutions that are not autoclaved, such as CPMU and starch solutions, may prevent environmental contamination from entering the biofilm during the setup, such as members of the *Enterococcus* genus. While these are commonly present in oral samples, they were significantly more abundant in our samples than the natural oral samples to which we compared them. Once changes to the model setup, the model will have to be re-validated, as the concentrations of nutrients, let alone the type of nutrients, will impact the community composition of the biofilms (Edlund et al., 2013).

We also used Fourier Transform Infrared (FTIR) spectroscopy to assess the mineral content of our model and compare it to natural dental calculus, both modern and archaeological. Our analysis showed that, after 25 days of growth, our biofilm model produced a substance that is chemically very similar to both modern and archaeological calculus. It is interesting that the mineral composition was so similar to natural calculus given the unique microbial profile. It suggests that the mineralisation occurs in a predictable manner regardless of the microbial profile, if conditions are favourable. Even in the absence of the known mineraliser, *Corynebacterium matruchotii*. The crystallinity of the model calculus also matched the archaeological sample we used as a comparison, though with a slightly less ordered structure. This may be related to the age differences in model calculus compared to archaeological calculus. Not only did the archaeological calculus spend a few hundred years maturing in the ground, allowing crystals to expand into the gaps created by degraded organic matter (Weiner, 2010), but given the known lack of oral hygiene practices in the past, the calculus was surely older than 25 days before being buried. We also only analysed a

single archaeological sample, so we don't know how representative this sample is of archaeological samples in general. Perhaps this was a particularly under- or over-mineralised sample. It would be more appropriate to compare to the modern reference samples, since we are actually trying to recreate something that mimics natural modern calculus, not something that has been buried for hundreds of years or more. Unfortunately we didn't have access to new modern samples and couldn't produce modern calculus grind curves for this analysis.

6.1.1 Model application

After establishing that our model dental calculus mimics, at least to some extent, the real deal, we assessed what biases may occur in starch incorporation. It is a mistake to think you can solve any major problems just with potatoes (Adams, 2002a), so we also included wheat starch in the model to cover a wider range of granule shapes and sizes. Put simply, we added a known amount of starch granules—well, to the extent we could estimate the large quantities in our starch solutions without counting every single granule—to our biofilm over the course of the 25-day experiment. Starch solutions were added on day nine of the experiment. This was a somewhat arbitrary decision; we only needed to ensure that there was enough separation between the last saliva donation and the introduction of starch treatments. We did this to prevent our starch counts from being affected by α-amylase activity from the donated saliva, thereby getting somewhat 'pure' counts from the added starches. However, we found no evidence of the model retaining α-amylase from the donated saliva, there is no reason the starch treatments couldn't start sooner in the experiment. For future experiments looking at the effect of amylase activity, it's important to still keep this under consideration, as amylase activity from natural saliva can fluctuate in individuals throughout the day based on both physical and psychological influences (Nater et al., 2005). Controlling the level of amylase activity in the experiment is more easily done with amylase artificiallsupplier of scientificy added to the model. Amylase can be purchased from your local supplier

of scientific equipment along with some overpriced sugar and baking soda. If it's not 'analytical grade' it's not

At the end of the experiment, we dissolved the calculus and counted the number of starches that were inside. Those who are familiar with previous dietary research on archaeological dental calculus will probably not be surprised that the number of starches we extracted was nowhere near the amount we put in. More interestingly, though, the size of the starch granules influenced the outcome; fewer large starches were extracted than what was put in the model during growth. This could be related to how starch granules are trapped in biofilms in the first place, where size and/or surface morphology of the starch granules could influence the likelihood of being retained in the biofilm. We also found that a very, VERY, low proportion of the starch granules that we 'fed' our samples actually made it into the dental calculus; only 0.06% to 0.16% of granules from the treatment solutions were extracted from the dental calculus (**Chapter 4**, Bartholdy & Henry (2022)). Given how few actually make it in, this may suggest that evidence for dietary starches are the result of repeated exposure to a large quantity of granule-containing foods.

6.1.2 Model limitations

So far I have covered what our biofilm model can do. It is equally important to talk about what our model can't do. After all, we demand rigidly defined areas of doubt and uncertainty (Adams, 2002c). While we have a high degree of control and reproducibility, especially when compared to *in vivo* models, there are certain conditions we cannot regulate with our current setup. This includes environmental conditions such as CO_2 and oxygen availability, which rely on the conditions in the lab where the experiments take place. To some extent, the bacterial communities within a biofilm can generate favorable conditions in a local environment through metabolic processes—one of the adaptive benefits from being part of a biofilm—but these are still somewhat dependent on the extrinsic environment in which they are situated. Biofilms on hard tissues will differ in

composition from those found on soft tissues. And biofilms found closer to the front of the mouth will differ from those found towards the back (Kolenbrander et al., 2010; Marsh, 2005; Palmer et al., 2003; Proctor et al., 2018). This difference is also something that is difficult to mimic in a single experimental setup; as is the ability to control salivary flow rates and circadian rhythms, both of which can influence the growth of plaque (Dawes, 1972; Proctor et al., 2018).

The effect of circadian differences in microbiome between individuals can influence replication of the microbial composition of our model, which will be limited by our use of whole saliva as inoculum rather than using a handful of select species. This means microbial profiles of the biofilms may change between (or even within) experiments, since the microbial composition of our saliva can vary slightly throughout the day, and the formation and composition therefore depends on the time of day the saliva is collected. It can also differ between donors. We reduced these limitations in our experiments by collecting samples from a single donor at the same time of day for each inoculation, but this will still cause differences between experiments.

The absence of α-amylase in our model may have affected the microbial composition of our biofilms. Our model has no renewable source for α-amylase once the inoculations have been completed. There are streptococcal species present in the model that are known for their ability to bind amylase (Haase et al., 2017; Nikitkova et al., 2013); however, we did not investigate whether the strains present in our model contain these genes. Starch solutions were only introduced on day 9 of the experiment. Prior to this, all samples were treated with the sucrose solution. The absence of starch during inoculation could have suppressed bacterial production of amylase-binding proteins (Nikitkova et al., 2012). Frequent medium replacements may also be clearing out all of the unbound host salivary amylase. We don't know exactly why α-amylase is absent, and need to look into this. In the meantime, this absence opens up opportunities to examine its role in the incorporation process of dietary materials (see below).

A well-known limitation of biofilm models in general is the difficulty in capturing the diversity and complexity of the natural oral biome. Diversity and complexity may be represented as interspecies communities and complex metabolic dependencies between organisms within the communities, or as an environmental complexity determined by nutrient availability, host immune-responses to biofilms, and fluctuating microenvironments across the biofilm in response to these factors (Bjarnsholt et al., 2013; Edlund et al., 2018). These limitations can be mitigated by complex experimental setups, but at the cost of lower throughput and higher financial cost. Increasing the number of species included in a model can approach the diversity found in the natural microbiome, but still falls short of capturing the complete diversity (Edlund et al., 2013), and the use of whole saliva introduces another set of limitations (as discussed above).

Then of course there's the inevitable limitation that we're dealing with a model. An attempt to recreate the real thing under controlled conditions, allowing us to test a variety of circumstances and see what the outcome might look like in the real world. These are generalisations that may not be comparable to any specific real-world case, but allow us to view and quantify processes that can be difficult to access in natural systems. The very isolated and controlled model setup also deviates from the natural conditions in our mouths. Many of the biofilm's natural predators are not present in our setup. Plaque is constantly at risk of removal by the tongue, salivary flow, oral hygiene practices, even the act of chewing—processes which help shape the biofilm (this is counterintuitive since they are processes of removal) (Shaw et al., 2004).

6.1.3 Further model validation

Going forward, we aim to further assess the validity of our model, as well as optimise the protocol. While we have established that our model is capable of forming a mineral composite comprising a largely oral microbiome, there are properties that we have yet to determine. Just because the bacteria in our model are identified as oral, doesn't mean they necessarily behave like communities

of natural oral bacteria. By determining the functional and metabolic profiles of the bacteria and communities within our model, we hope to get further insights on metabolic dependencies, production of metabolic by products, and gene expression in our model. As a result we will be able to further optimise the protocol to more closely mimic the natural oral biome.

There are also other conditions within our model that we need to determine, such as monitoring physiological responses to changing conditions. For example, after carbohydrates have been consumed, there is a dip in the pH within the oral cavity as the carbohydrates are consumed by bacteria, which release acidic by-products. This occurs within the first few hours of consuming carbohydrates, after which the saliva will work to balance the pH back to pre-carbohydrate levels, also known as the 'Stephan curve' (Stephan & Hemmens, 1947). By acting as a buffer and restoring the oral pH-level, saliva can help prevent high levels of acid from demineralising the tooth surface and causing caries. Since our model is fed both with sucrose and starch, it is important to know that the pH levels don't permanently drop to levels that are unfavourable to mineral supersaturation and plaque mineralisation.

Since FTIR only addresses the overall mineral composition, we will need to further investigate whether there are any other structural/chemical differences between our model and natural calculus that may be caused by microbial profiles, and microscopically examine the model to determine the micro-architecture.

6.1.4 Potential biofilm model applications in archaeology

Biofilm models are an untapped resource in archaeological research, especially for dental calculus research. Coupled with existing validation methods to address current dental calculus limitations, the proverbial sky is the limit. This section describes some possible archaeological applications for a biofilm model, but is certainly not complete. It is mainly comprised of questions that arose during the experiments I conducted, as well as during the analysis of archaeological material, that I was unable to address in this dissertation due to time

constraints. Hopefully these questions can be answered by myself or others in the future.

The main question that came up during experiments concerns the mechanism of incorporation of dietary compounds, especially starch granules, in dental calculus. How does it actually happen? This seemingly simple question is particularly challenging, and one that I hadn't prepared for in my experimental design. Going forward it will be an important question to answer, as it may influence the likelihood of certain compounds to become trapped in dental calculus, and at what point during the formation and mineralisation process this occurs. By staggering the treatments during the experiment, we may be able to see if the rate of incorporation varies during biofilm growth, and whether or not particles can penetrate the surface of the calculus after it has mineralised. If not, this could mean the layered structure is indicative of chronological consumption events. If so, what is the size limit? Can starches infiltrate dental calculus post-burial, or is this limited to smaller molecules? And do the chemical/physical properties of molecules and microremains (amylopectin content of starch granules, polarity and hydrophobicity of molecules, etc) influence their ability to become incorporated or penetrate the mineralised surface? This question of incorporation also came up during the analysis of archaeological dental calculus in **Chapter 5** (Bartholdy, Hasselstrøm, et al., 2023). Based on the presence of many metabolites, it seems that this may not have been during consumption, but rather during excretion through saliva, or, put more simply, when the molecules are on their way out of the body again. This makes some sense, since food actually spends relatively little time in our mouth while we're eating, and significantly longer travelling through our body. This may also explain the very low retention of starch granules we found in **Chapter 4**. It seems that most of the starch granules are swallowed, while few become lodged in our teeth/plaque and are eventually trapped in dental calculus. Without looking into the mechanism by which starches and other food molecules are incorporated into dental plaque, we are always going to be guessing (albeit educated guesses) what is happening archaeologically.

An important question to address within the framework of incorporation pathways, is what role bacteria play in the incorporation of dietary material, and whether differing bacterial profiles have an impact on the retention of dietary molecules and microremains. It is likely that they will cause differential retention given that they make use of a lot of the food that passes through our mouths with the help of digestive enzymes (Rogers et al., 2001). The important question to answer is how, and, to what extent, they influence this process. A systematic approach would be to set up multiple experiments with different sets of defined consortia grown under the same conditions. On a related note, the absence of host salivary α-amylase activity in our model (as shown in **Chapter 4**, Bartholdy & Henry (2022)) provides an opportunity to explore the effect of various amylase levels on the incorporation and retention of dietary compounds, especially starches, in dental calculus. Alpha-amylase can be purchased from most laboratory supply companies, and can therefore be added to the model and explored as a controlled variable. Some bacteria have the ability to bind α-amylase in order to use the degradation products of starches as nutrients (Nikitkova et al., 2012; Rogers et al., 2001), so the abundance of these bacteria coupled with α-amylase activity will likely influence starch retention.

Finally, it's worth noting how important it is to be able to generate an unlimited number of samples for validating current methods and developing new ones. Archaeological dental calculus is a finite material and should be treated as such. We should know exactly what we're doing when we are analysing samples. If not, then model dental calculus would be a great substance to try out new things, and even for training researchers on the range of methods at our disposal.

6.2 Dental calculus in archaeology and future challenges

Dental calculus has provided unique perspectives on multiple activities of humans in the past, from dietary practices to the evolution of the oral microbiome.

Researchers continue to find innovative ways to extract information from a material that was once discarded. It is uniquely situated to address diet because of its direct interaction with everything that enters (and exits) our mouth, some of which leaves clues behind that are embedded within the calculus itself. There are, however, still limitations to address to further unlock the potential of dental calculus to reconstruct past dietary activities. Probably the main challenge we face in archaeology, let alone studies of dental calculus, is identifying contamination versus the authentic remains left behind from the past. A challenge more specifically related to dental calculus, is understanding why some things are retained in dental calculus, and why others are not. Finally, we should continue to optimise our sampling and analytical methods to make sure we are getting the most out of these small deposits of minerals, bacteria, food debris, and whatever else made its way into the mouth during life.

6.2.1 Incorporation pathways

As discussed above, one of the main challenges of working with dental calculus is our lack of understanding of incorporation pathways. We need to know how exogenous material becomes trapped inside, and to what extent the processes within the oral cavity cause damage to, or completely eliminate, the dietary compounds.

The incorporation pathway for larger particles (relatively speaking), such as dietary starches and phytoliths, is likely during consumption of foods that contain them. What exactly about their morphology or physicochemical properties allows them to enter and become trapped is still unknown. The surfaces of starch granules mainly contain polar phospholipids (Cornejo-Ramírez et al., 2018), making the phospholipid bilayer of a starch granule compatible with, or even attracted to, a biofilm consisting largely of water. Conversely, hydrophobic molecules might be less likely to associate with a biofilm, and therefore be underrepresented in any analysis on dental calculus, if they are present at all. Once starch granules become attached, the repeated process of biofilm growth

would result in the starch molecules becoming trapped between two biofilm layers, increasing the likelihood of retention. Once trapped inside the biofilm, retention of the dietary particles depends on the ability to avoid digestive enzymes that are commonly used by the communities of bacteria to break down the macromolecules into more manageable sizes. This gap in our knowledge is also why we don't understand why the remains of some plant species are overrepresented while others are underrepresented. We know that this happens, but not why. Smaller molecules may be able to hitch a ride through diffusion channels that transport nutrients into the biofilm (Flemming & Wingender, 2010), although biofilms are known for their ability to limit diffusion of specific molecules, such as antibiotics (Stewart, 2015). Diffusion of molecules has been explored clinically, but mainly focusing on antibacterial agents (R. Ma et al., 2010; Stewart, 2015; Takenaka et al., 2009). So far nothing has been done to explore the dietary perspective in which we're interested.

The incorporation pathway may also be heavily influenced by mode of consumption. If someone was chewing tobacco or storing coca in their cheeks, the most likely place to detect nicotine or cocaine, the principal alkaloids of these plants, would be in dental calculus deposits on the molars. However, mucous-rich saliva, produced by the sublingual and submandibular glands (located in the front of the mouth), preferentially binds toxins (Dodds et al., 2005), making the anterior teeth a good hypothetical target for detecting these compounds.

Another potential pathway is the presence of molecules in dental calculus as a result of excretion from the body through the saliva. If you consider the amount of time you spend with food (or other things) in your mouth, it is relatively short. A few minutes at most? Whereas the time spent in your body is much longer, as food molecules enter the bloodstream and are distributed throughout the body. The molecules can then re-enter the mouth through the saliva and spend significantly more time in the mouth the second time around, as excretion may take days (Lee et al., 2011). At this point the original compounds may have been broken down by, for example, the liver or kidneys, in which case mainly

the metabolites will be present. The plausibility of finding molecules via this pathway depends on the size of the molecules and the ability to diffuse from serum/plasma to saliva and enter the oral cavity. Given this incorporation pathway, the molecules are, hypothetically, more likely to be secreted in higher concentrations through the serum-rich saliva of the parotid glands, located next to the molars (Dodds et al., 2005). Molecules originating from this pathway would mean that it, unfortunately, wouldn't be possible to determine the mode of consumption (e.g. chewing vs. smoking) based on the mass spectrometric results alone, but would require further analysis of the dentition to identify. For example, if nicotine is detected, it would be useful to identify tooth staining and periodontal disease caused by tobacco smoking (Ness et al., 1977). It would also require relying on contextual materials found at the site, but that's something which should be done anyway. To bridge this essential gap in our knowledge, further testing through systematic sampling of different parts of the dentition is needed.

6.2.2 Identification of fragmented remains

Identifying and quantifying plant microremains has a particular set of challenges, even before the food has entered our mouth. Humans have become reliant on processing foods to aid digestion and to maximise the energy acquired from eating. Unfortunately, this also means that the microremains are put through various damaging processes during preparation (García-Granero, 2020). Pre-cooking processing may already render starch granules unidentifiable (Li et al., 2020). During cooking, starch granules are, at best, modified and, at worst, completely destroyed depending on the cooking method (Henry et al., 2009). The granules that survive the cooking process are then submitted to further harm in the oral cavity by the act of chewing and the presence of digestive enzymes. After death, the starch granules that are trapped in dental calculus will have to resist degradation from the burial environment, including bacteria, fungi, and water damage (García-Granero, 2020). To add final insult to

injury, further damage can occur during excavation and processing of the dental calculus (Tromp et al., 2017), and even during preparation for microscopic identification (García-Granero, 2020). Through all this, there are still dietary molecules and microremains that somehow survive hundreds-to-thousands of years inside dental calculus, and remain identifiable. Our next challenge is to determine how to interpret these remaining microremains. To date, most experimental methods have addressed the damage and modifications occurring to microremains present on tools and cooking utensils (Langejans, 2010; Li et al., 2020; Z. Ma et al., 2019), and not in the context of dental calculus. Given the added processes affecting the survival and morphology of microremains unique to the oral cavity, this context is very important. Validation conducted on archaeological remains will suffer from the same limitations as *in vivo* studies, namely the variability of dental calculus growth. The variability can affect comparisons between two or more individuals, as well as between dental calculus deposits within the oral cavity of a single individual. The human oral cavity is home to many unique environments causing differences in the chemical and bacterial makeup of dental calculus (Fagernäs et al., 2022; Hayashizaki et al., 2008). Our best option to control these many factors and explore the precise nature of their individual impact on the incorporation and retention of dietary materials in dental calculus, is to isolate these factors in separate, controlled experiments in a lab.

Methods developed more recently offer us the ability to make identifications on a much smaller scale. The 'omics' approaches can be used to detect many compounds which are otherwise invisible to the naked, microscopically-aided, eye. There are still limitations to these methods. Ancient DNA (aDNA) and paleoproteomics are limited by the low amount of diet-related genetic material present in dental calculus compared to an overwhelming number of host-associated genomes related to the millions of microbes inhabiting the oral cavity. Further complicating the matter is the inability to assign damaged DNA sequences to a single precise species designation, and instead relying on low resolution estimates (Mann et al., 2023). Similar issues are encountered in protein identifica-

tion (Hendy, 2021).

Adding to the challenge is the fact that not all materials will degrade in a similar manner. Some materials/molecules are more robust than others. To what extent, then, can we interpret the difference between the abundance, or even presence and absence, of materials detected within and between individuals? We know that the stability of molecules plays a role in what will ultimately be detectable by mass spectrometry. The chances of finding principal pharmacologically active or psychoactive constituents of plants, such as morphine or tetrahydrocannabinol, are relatively slim since these molecules are unstable and have a hard enough time surviving decades, let alone (pre-)historic timescales (Lindholst, 2010). Protein and bacterial abundances are also impacted by differential degradation (Hendy, 2021). This makes it hard to determine whether the quantities of molecules are an accurate reflection of the quantities during life, which in turn complicates interpretations we make on the health and diet of individuals.

6.2.3 Contamination and lab processing

It has been shown that dental calculus preserves well, and that little external contamination enters the calculus after burial (Warinner, Rodrigues, et al., 2014). Dental calculus is a robust material. After all, it's made from a lot of the same material as bone. It can clearly provide good protection to the microremains and various molecules trapped inside, and survive thousands of years (Fellows Yates et al., 2021; Henry et al., 2014). It is, however, not impenetrable. In fact, it can be quite porous (Friskopp & Hammarström, 1980; Robert C. Power et al., 2022). This means it's important to consider what may have been originally trapped within the calculus during life, and what could have entered postmortem. The proportions of original to exogenous material may also change with time, depending on the physicochemical properties of the molecules. It seems that small hydrophilic molecules are more often lost from dental calculus than larger hydrophobic molecules, suggesting postmortem movement of

water through the substrate (Velsko et al., 2017). In addition, these molecules may also be present as contamination in labs or in the burial environment. I cannot stress enough how important it is to collect control samples from surrounding soil and to replicate findings in separate labs, with clear identification of potential contaminants (Crowther et al., 2014).

In the study from **Chapter 5** (Bartholdy, Hasselstrøm, et al., 2023), we detected various compounds in dental calculus using UHPLC-MS/MS, including salicylic acid, a phytohormone from willow trees (*Salix alba*, for example) with medicinal properties. Willow bark has long been known for its medicinal properties, and is present in many common foods. It is therefore not surprising that we found it in the dental calculus of people from the 19th century. We also know, however, that salicylic acid is abundant and very mobile in soil. With this in mind, how do we interpret our findings? There are currently no standards for authenticating results from GC/LC-MS/MS analyses on archaeological samples. Research in aDNA uses, among other things, damage patterns from the sequences to determine whether a sequence is old or not, and there are many tools available to accomplish this, such as decontam (Davis et al., 2018), PMD tools (Skoglund et al., 2014), HOPS (Hübler et al., 2019), and cuperdec (Fellows Yates et al., 2021). Similarly paleoproteomic research can look at markers of degradation, such as deamidation (Ramsøe et al., 2020). We attempted to provide a method to authenticate our finds by plotting the quantity of compounds in three washes and comparing these quantities with the quantity extracted from the calculus itself. We expect to see a decrease in quantities over the three washes as surface contaminants are removed, and a subsequent increase in quantity as the calculus is dissolved and the compounds that were embedded within the calculus are extracted (Bartholdy, Hasselstrøm, et al., 2023). This assumes that the embedded compounds were incorporated during life, and does not in any way verify that the molecules are actually old. So what does this mean for our interpretations? Until we can find a way to separate external contamination from authentic compounds from the past, and quantify the extent of external contamination in dental calculus, we can say that they most likely consumed plants containing

salicylic acid, but that we also cannot rule out contamination from the burial environment as a source. It's most likely a combination of both.

We also included modern synthetic compounds that we know would not have been present in the past. These included MDMA, Fentanyl, Amphetamine, and others. We detected cocaine in nine individuals. Cocaine is not a modern compound, since it has been used for millennia in the Americas (Abduca, 2019; Indriati & Buikstra, 2001; Springfield et al., 1993), however, it didn't become known to Europeans until colonisation in the late 15th century, and was only widely adopted in the late 19th century after cocaine was isolated by Albert Niemann (Abduca, 2019; Company, 1886). This complicated things. Cocaine is an alkaloid found naturally in the leaves of various species of coca plants. While we wouldn't expect a rural population from 19th century Netherlands to have access to coca leaves, it wasn't impossible to imagine. It was commonly observed to prevent fatigue and suppress appetite, potentially useful to farmers. There was some Dutch presence in South America with the Dutch West Indies, and they even established the *Nederlandsche Cocainefabriek* in Amsterdam in 1900 (Bos, 2006). Given the possible impact of such a finding, we analysed new samples from the same individuals in a separate lab on different equipment. We were unable to detect cocaine in any of the replicated individuals, and it was probably a case of some sort of lab contamination that managed to slip past our blanks (Bartholdy, Hasselstrøm, et al., 2023). Upon further research, we were unable to find historic evidence of coca leaf-use in Europe for anything other than study, and the only small-scale botanical imports were recorded prior to the late 19th century (the most recent individuals included in our study were buried in the 1860s). Coca leaves are also susceptible to decay during travel and may not have been viable for their intended use once they arrived in Europe (Abduca, 2019).

Contamination is widely recognised as a risk in all aspects of archaeological research, including paleobotany (Crowther et al., 2014) and aDNA (Cooper & Poinar, 2000; Gilbert, Rudbeck, et al., 2005; Gilbert, Bandelt, et al., 2005; Knapp

et al., 2012; Llamas et al., 2017), often because of bold claims made in the past (no specifics will be mentioned here). Protocols for dental calculus sampling include various steps for decontaminating dental calculus, and range from brushing the surface to UV-radiation and sonication. However, the use of liquids for decontamination may be problematic when there are plans to do biomolecular analyses (Velsko et al., 2017). Sodium hydroxide (NaOH) has been suggested as a better decontamination solution based on testing on synthetic precipitates of calcium phosphate (the principal component of dental calculus) (Soto et al., 2019). It's not clear how valid this approach is since the synthetic dental calculus was grown without bacteria, and they're generally responsible for the channels (supplying nutrients) in dental calculus that would allow a decontaminating agent to seep into the calculus and affect the microremains. Nevertheless, it is a step in the right direction.

After decontamination, the dental calculus is dissolved to extract the remains trapped inside. The exact method for dissolving dental calculus inside depends on the type of analysis being done. The most commonly used chemicals for extracting starches from dental calculus are hydrochloric acid (HCl) and ethylenediaminetetratetraacetic acid (EDTA). HCl has long been the preferred method for decalcification of dental calculus for extraction of plant microremains (Hardy et al., 2016, 2018). However, there was no apparent testing on the original use of HCl (Middleton, 1990), which was originally developed for extraction of phytoliths, which are very resistant to chemical degradation (Cabanes, 2020). It has since become clear that dental calculus is also a rich source of starch granules (Henry & Piperno, 2008; Scott Cummings & Magennis, 1997), though it's not entirely clear how resistant starch granules are to degradation by acids. It was briefly mentioned in Henry & Piperno (2008) that weak solutions of HCl would not affect starch granules, but more recent research suggests that EDTA can recover more material from archaeological dental calculus than HCl (Tromp et al., 2017) and cause less damage to the starches (Le Moyne & Crowther, 2021). Validation of methods on archaeological material is difficult since we don't really know the starting point.

One way to explore the external contamination of calculus and how it may affect already present compounds and microremains, is to set up an experiment where model calculus samples containing known quantities of compounds (and controls without anything) are buried for different periods of time (within a reasonable timeframe). We originally attempted this, but the model calculus protocol was not ready and the model calculus samples were not sufficiently mineralised to survive in the ground. The initial biofilm growth and burial are included in a blog post (https://www.leidenarchaeologyblog.nl/articles/spit-tartar-and-burial-an-experiments-diary), but no further results were written up because of the aforementioned issue with the protocol, and intrusion by a pandemic. This particular failure motivated me to revise the protocol and properly validate the grown model dental calculus (see **Chapter 3** and Bartholdy, Velsko, et al. (2023)).

There is an art, or rather, a knack to decontamination and dissolution of dental calculus. The knack lies in learning how to make sure all contaminants are removed and authentic material is dislodged from the minerals, and preventing further degradation of the authentic materials crucial to our understanding of past dietary activities. To continue the laboured analogy from the beginning of this chapter; we don't want to cause any more damage to the already broken puzzle pieces. Since it's clear that water can potentially clear out some of the original molecules from dental calculus, we need to be careful with lab cleaning and processing methods, and more extensive research on the effects of processing methods needs to be done.

6.2.4 Deliberate and efficient sampling and analysis

Dental calculus has many advantages over other elements from skeletal remains, especially when it comes to dietary reconstructions. With dental calculus we can more reliably argue that the substances we find within are the result of direct consumption. Dental calculus is, after all, formed inside our mouth, which is, famously, used during the act of eating. It would be hard to justify the presence

of plant microremains found on any other element of skeletal remains as a result of consumption. Any starches found outside of dental calculus, even within the enamel of teeth, would likely have gotten there after death as the result of environmental contamination. This doesn't mean we can throw caution to the wind and interpret everything in dental calculus as food (Radini et al., 2017), but it is one of the likelier scenarios.

Because the formation of dental calculus is continuous throughout life, the information we extract about diet more likely reflects a broader time frame, but given the potential for many growth disruptions and removal, it probably reflects dietary patterns closer to the individual's death (depending on the size of the deposit). That being said, other skeletal elements also have advantages over dental calculus that should be considered when studying diet. When it comes to studying the childhood of adult individuals, dental calculus would not be applicable. This is because of the aforementioned cycle of potential mechanical disruptions, and the fact that dental calculus is uncommon in younger individuals. Any calculus visible on an adult skeleton is unlikely to have formed during childhood. Here, enamel represents the most appropriate choice. Enamel is formed during childhood and remains largely unchanged during life (Hillson, 1996), so any dietary influences from childhood during the time of enamel formation, which spans around 28 weeks *in utero* to around 16 years (*ex utero*, of course) (Hillson, 1996), will be present in the enamel of the adult dentition. Similarly, bone and dentine (depending on where you sample the dentine) have a slower turnover, and represent a more stable source of dietary patterns. And since they are generally not exposed to environmental contamination during life (otherwise you're in trouble), they may, in some cases, be more reliable. However, methods using these skeletal elements suffer from a low resolution, since they can generally "only" (highly exaggerated air quotes since it's still incredibly useful) offer insights into very broad dietary trends (Katzenberg, 2008), whereas methods used on dental calculus can be much more specific, sometimes even incredibly so (Hendy et al., 2018; Scott et al., 2021). Others have also noted that the source of collagen protein in dental calculus, the primary target for stable

isotope analyses, can be difficult to determine given all the microorganisms residing in plaque and dental calculus. This leaves questions about what the isotopes are actually saying about diet, if anything (Price et al., 2018; Salazar-García et al., 2014), and may be more related to dental disease or contamination from other archaeological materials (Mackie et al., 2017).

If sheer quantity of DNA is what you're after then there really is no better substance than dental calculus. It is estimated to contain up to 170 times more DNA in archaeological samples compared to dentine samples from the same tooth. The main difference is the presence of microbial DNA. For human host DNA, the abundance in dentine is typically higher, though more variable. Dental calculus contains limited host DNA, which may be difficult to capture given the lower relative abundance compared than bacterial DNA, and it can be more fragmented (Mann et al., 2018; Ziesemer et al., 2018). This difference is due to the nature of the two substances. During life, plaque is primarily made up of bacteria, while dentine does not contain any bacteria. The exception is in some cases of oral disease, such as periodontitis, where the presence of bacteria is a byproduct of the disease process. Since dental calculus is also a trap for food debris, dental calculus can contain plant DNA and food proteins (Fagernäs et al., 2022; Hendy et al., 2018; Scott et al., 2021; Warinner, Hendy, et al., 2014). The problem with detecting dietary DNA in dental calculus is the same as for human host DNA; there is very little of it, and it may be highly damaged. This causes problems when trying to identify the source of the DNA. If the DNA sequences are not long enough to distinguish between multiple related sources (e.g. mammals), then interpretations can be made difficult (Mann et al., 2023). That being said, as our techniques develop and we accumulate more complete reference databases that allow us to make more robust identifications on smaller DNA fragments, dental calculus can become even more of a treasure trove of information than it is already.

Detecting metabolites in dental calculus has its own set of considerations. Until now, the most common separation method for analysing metabolites has been

using high temperatures to vaporise samples into a gas phase (the 'GC' in GC-MS) and decompose metabolites within samples for subsequent identification by mass spectrometry (MS). The benefit being large reference databases used to identify various compounds. However, it may not be the best option for every use-case, and the high temperatures required can cause problems, such as degradation of the compounds. Some metabolites, particularly alkaloids, are less volatile, and are therefore not easily vaporised and detected following derivatization (Zimmerman & Tushingham, 2023). This is not a great feature when looking for potentially interesting dietary and non-dietary alkaloids. Methods using liquid chromatography coupled with mass-spectrometry (LC-MS) use lower temperatures and are able to detect these compounds directly, without the step of derivatization (Rustichelli et al., 1996; Sørensen & Hasselstrøm, 2017). This reduces sample preparation time, but comes at a higher cost, financially for instrumentation and operators (a serious consideration for archaeological budgets).

If dental calculus is the best substance for the particular research goal, then it's important to maximise the information extracted from the samples, and minimise the amount of sample needed. Since dental calculus has become the target for many different types of analyses and studies, there have been attempts to unify extraction protocols for different analyses to save on time and minimise destructive sampling, such as a combined extraction protocol for aDNA and proteomics (Fagernäs et al., 2020) and aDNA and plant microremains (Modi et al., 2020). The sequence of analyses should also be considered, as some 'non-destructive' techniques may cause invisible damage to the samples. For example, high-powered imaging techniques involving radiation may affect the quantity and quality of extracted DNA (Immel et al., 2016). We should continue to explore ways to minimise the amount of material required to conduct our studies.

While they are abundant in the past, dental calculus deposits are quite small, ranging from less than one to around a hundred milligrams. It is therefore im-

portant to make our sampling as efficient as possible so we can retain some of the material for future analyses and replication. Many of the analytical methods used on dental calculus required destruction of at least part of the sample. When deciding to perform destructive analyses, it is important to consider the goal of the research. Dental calculus may not be suitable for all purposes. It's important to select the right tool for the job. There are likely better sites on the human body to sample for human DNA. And while it has been preferentially targeted due to the fact that it's technically considered an ectopic growth and is not given the same ethical scrutiny as skeletal material, maybe it should. After all, it does contain human DNA, and our microbiomes are unique.

6.3 Thoughts on the future

It's hard to imagine the future of dental calculus to be anywhere else than in the hands of biomolecular methods. Further refinement of our methods will identify and address current weaknesses and improve our interpretations. Such method validation should be performed on a model with known input, to accurately assess the outcomes and biases of our analytical methods. Something that cannot be achieved using archaeological dental calculus. By validating what we see in an artificial substrate with known input, we can accelerate our knowledge and start to make bolder interpretations that are grounded in systematic experimentation.

A model can provide insights on many of the challenges listed above, including differential degradation of remains (starches, metabolites, DNA, proteins, etc.), likelihood of incorporation and retention during life. What does it mean when we find X number of potato starches and Y number of grass phytoliths in dental calculus? What does it mean when we detect certain ratios of metabolites and can we use that to identify a source? Model calculus is potentially a useful material to test the recovery rates of unified protocols compared to separating samples and analyses. Using robust materials as a control, it would be possible to track

the process from incorporation to extraction and quantification without worrying about what was lost to enzymatic and acidic damage. An example of such a material is palynospheres, black ceramic spheres which are used as marker grains because they are resistant to chemical and mechanical degradation. They were created as an alternative to *Lycopodium* spore tablets in places where you might expect to find indigenous *Lycopodium* spores (Kitaba & Nakagawa, 2017).

The wide range of analytical methods that can provide important insights on dental calculus require a similarly wide range of expertise. Inter-disciplinary collaboration is an absolute must for analyses involving a deep understanding of scientific methods, as well as continuous communication between archaeologists and other fields to understand the limitations and strengths of methods and interpretations in an archaeological context. Lists of authors on archaeological papers are growing; as they should. Paleoproteomics has already shown that it's possible to detect very specific information about dietary molecules present in dental calculus, down to the type of food, its source, and method of processing (Hendy et al., 2018). It also has the advantage over DNA in that proteins seem to preserve for longer. Further development of reference databases and analytical methods is continuously improving the fields of paleoproteomics and (oral) metagenomics by increasing quantity of, and confidence in, species identifications of dietary sources and improved methods for authenticating truly ancient sources of materials. It will be exciting to see where these fields can lead us as they mature.

Another area which may lead to exciting discoveries is accessing the layered structure of dental calculus through high-powered imaging techniques (e.g. Robert C. Power et al., 2022). We know that the formation of a biofilm is sequential, with new layers of biofilm continuously forming on the already established layers. Sequential analysis of dental calculus layers might therefore be able to determine a sequence of incorporation events for dietary material in dental calculus. However, since we can't yet access information about the age of occurrence of the seemingly haphazard mineralisation events in dental plaque, it

is difficult to envision a scenario where we can talk about dietary activities and the age of individuals. Until then, though, it will still be beneficial to be able to generate a sequence of deposition events and talk about the dietary material found in each layer.

Amidst a scientific revolution, it's important to remember that there are things that can be said about dental calculus without using biomolecular or microscopic methods. Not to mention, visually scoring calculus deposits is cheaper and requires no specialised equipment. The presence of dental calculus and the size of the deposit can be meaningful. For example, Yaussy & DeWitte (2019) found a decreased survivorship in individuals with dental calculus formations. Past populations are also a well-suited target to explore the relationship between dental diseases, such as dental calculus and periodontitis; and between dental diseases and diet, since oral hygiene interventions were less widespread in the past. Therefore, it's crucial to record the deposit *in situ* before proceeding with destructive sampling. This means taking photos and scoring the deposit using existing methods, such as (Brothwell, 1981), and recording detailed information allowing researchers to filter out unnecessary information in downstream analyses rather than missing out on something that was never recorded. Ideally, each surface of the tooth should be scored separately to retain the most information for future analyses, and allows calculating a dental calculus index (Greene et al., 2005). Calculating an index with calculus scored on multiple surfaces of the teeth allows us to reveal more patterns related to the presence and absence of dental calculus, such as uneven distribution within the dental arcade, allowing more fine-grained comparisons between populations and within different groups in the same population. No analytical method should be considered the be-all and end-all of our analytical toolkit. Results should not be considered in isolation. The best approach considers multiple angles and makes use of multiple lines of evidence to reach robust interpretations. Not only multiomic approaches, but studies that incorporate the entire spectrum of archaeological analyses.

Working with new scientific methods and improving our analytical approaches is only one small way to contribute to existing knowledge of dental calculus, and may be unproductive if the method has already been tested by other labs. Moving past the disregard for 'null' results will prevent researchers from conducting the same experiments (as other labs) and expecting a different result. Registered Reports allow researchers to apply a method and have it guaranteed to be published in a journal, not because the results were deemed "positive" or "novel", but because their methodology was sound and their results contribute to a robust, scientific foundation of knowledge (Chambers et al., 2014; Nosek & Lakens, 2014). Opening our methods will facilitate faster improvements to existing protocols, as well as open up opportunities for researchers in smaller labs. Here I'm not talking about vague, cryptic methods sections in papers, but detailed protocols accessible to anyone with the necessary materials and equipment. Platforms like protocols.io are a great solution (e.g. 10.17504/protocols.io.bvt9n6r6 and 10.17504/protocols.io.dm6gpj9rdgzp/v1). Adopting more open research practices will also make it easier to incorporate multiple proxies in research studies, as this will no longer be limited to those with access to enough material and range of materials to conduct large-scale analyses (such as Fellows Yates et al., 2021). Ensuring that we publish our data in a manner that is Findable, Accessible, Interoperable, and Reusable (FAIR) will promote reproducibility and replication, two crucial aspects of scientific research (Wilkinson et al., 2016). Creating communities that can promote these practices within specific fields and subfields can be effective in creating relevant standards and fostering an environment that promotes equitable research practices. This has been realised by the SPAAM community and Open Phytoliths with AncientMetagenomeDir and the FAIR Phytoliths Project, respectively. Unfortunately, many of these initiatives fall on researchers early in their careers out of a need for more resources or sheer enthusiasm for what they do. There are still very few incentives for organising these resources and practicing Open Science, and instead rewarding fast science and measures of impact that have somehow been assigned as important. Out-dated reward systems are preventing the

widespread adoption of open practices and disproportionately impacting young scholars and early career researchers.

6.4 Concluding remarks

In my dissertation I set out to put dental calculus under the microscope, scrutinising what we know about dental calculus, what we think we know, and what we need to know. To do this I created a model system that allowed me, and will allow myself and others, to address fundamental processes involved in all aspects relevant to the dental calculus analytical lifecycle. Processes including formation and growth, exposure to dietary and non-dietary materials, burial with subsequent degradation of original materials and the colonisation of materials and molecules from the burial environment, decontamination and extraction of materials trapped within the calculus, and many more. With the help of co-authors, the model dental calculus was examined to ensure that the bacterial and mineral compositions were sufficient to mimic an oral environment and closely resemble natural dental calculus. We deemed this to be satisfactory, but further validation is absolutely encouraged. The model calculus system was put into action to see what it could contribute to the use of methods to extract and quantify plant microremains from dental calculus. It showed that there is more to the process than dietary input, and that size, morphology, and physicochemical properties of granules may have an impact on what we ultimately end up seeing in archaeological dental calculus. We applied a new method, previously validated on cadavers, to explore the use of dietary and non-dietary alkaloids and metabolites in a rural Dutch population from the 19th century. Detection of mundane everyday compounds, such as those present in tea and coffee, has never been more exciting! Even the absence of compounds raises a number of questions about why they were absent, and if they were ever there to begin with. Contamination is omnipresent in archaeological studies, especially those employing biomolecular methods. Ours was no exception, with the possible, but unlikely, detection of cocaine. Overall there were more questions generated dur-

ing the various projects than I could possibly hope to answer over the duration of a PhD program (plus a little extra), and there is a clear need to address many challenges going forward, some of which may be addressed with oral biofilm models.

I have no doubt that we have just scratched the surface of what dental calculus can do to inform us about past activities, diet and otherwise. Novel analyses and biomolecular techniques have already taken us beyond what was likely imagined back when archaeological dental calculus was discarded. Microscopy, metagenomics, and paleoproteomics have already provided incredibly detailed insights into the dietary activities of people in the past, and will undoubtedly continue to improve our understanding. Before we can achieve any of these things, though, we need to take a closer look at how dental calculus incorporates these markers of diet, and what biases the mechanisms of incorporation may cause. Advances in dental calculus and dietary reconstructions will require a deeper understanding of the substance. How it behaves under certain conditions and how it interacts with the material and environments with which it comes into contact. This dissertation provides one possible solution to the need for more fundamental research required to understand these processes, adding to our toolkit of method-validation, which already includes ethnographic research, and experimental archaeology.

We already understand that we are limited in what we can say about diet in the past from dental calculus, especially from a quantitative perspective. It's not enough to identify the problems, but rather to identify the causes of the problems and their implications. With more systematic research answering more fundamental questions, maybe we can move beyond these limitations and be a little bolder in our interpretations. How can we possibly expect to understand diet from archaeological dental calculus if we don't understand fundamental processes that lead to dietary components ending up in dental calculus in the first place? Basically, we need to ask more stupid questions. They are probably not stupid; it's more likely that they point out fundamental assumptions that we

have been making without actually going through the trouble of testing them. After all, "You can't possibly be a scientist if you mind people thinking that you're a fool" (Adams, 2002b).

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Supplementary Information

This dissertation contains a lot of supplementary materials. Therefore, none of it is included in the dissertation itself. I will instead provide links to the online material, which will be useful unless you are reading a physical copy. If you are reading the physical book, well done—it means I like you enough, or felt obligated enough, to provide you with a rare, albeit valueless, copy. Or mabe you have borrowed a copy of this VERY niche PhD dissertation from the library. In both cases you have to options: you could tediously type out the entire url, or you could just go read the online version.

Most of the materials can be found on the Open Science Framework (DOI: 10.17605/OSF.IO/3YX8M).

The oral biofilm model protocols are published on protocols.io (DOI: 10.17504/protocols.io.dm6gpj9rdgzp/v1).

Article 1:

- **DOI:** 10.1101/2023.05.23.541904
- **Supplementary material:** 10.1101/2023.05.23.541904
- **Code:** 10.4121/99932661-fe79-4f4e-a812-a8917ad18fd0
- **Data:** PRJEB61886 (ENA accession number) & 10.4121/466b2588-9689-4d84-a8a0-5216aa39e40b

Article 2:

- **DOI:** 10.3389/feart.2022.886512
- **Supplementary material:** https://www.frontiersin.org/articles/file/downloadfile/886512_supplementary-materials_datasheets_1_pdf/octet-stream/Data%20Sheet%201.PDF/1/886512?isPublishedV2=False
- **Code and data:** 10.5281/zenodo.5604669

Article 3:

- **DOI:** 10.24072/pcjournal.414
- **Supplementary material:** 10.5281/zenodo.10069669
- **Code:** 10.5281/zenodo.11040640
- **Data:** 10.5281/zenodo.7648756
- **PCI_Archaeology recommendation:** 10.24072/pci.archaeo.100389

Summary

Dental calculus. This small, hard, inconspicuous substance that forms on the teeth of humans and animals contains a surprising amount of information about our lives. During formation and growth as a living plaque biofilm, it tends to accumulate a wide variety of very small particles, especially bacteria and food debris, from various sources. These sources and the particles they leave behind in our mouth are influenced by activities and biological processes that are unique to us, such as our dietary preferences, oral hygiene practices, genetics, and the environment in which we live. What makes it so interesting to archaeologists is that, following mineralisation, these particles become trapped and well-protected against removal and degradation during hundreds to thousands of years in the ground, preserving a picture of the activities performed by its human host. This picture can be unlocked by archaeologists by extracting and identifying proteins from plants and animals, and genetic material and microremains from plants that were trapped inside the calculus matrix. The major problem—one of the major problems, for there are several—one of the many major problems is that this picture was never a complete picture of a lifetime of activities. Another problem is that it has faded over the years, and some parts of the picture have been completely erased. There are many things influencing what gets trapped inside dental calculus, what gets preserved for all those years until it can be analysed, and how much of that information we can extract

and interpret. We know that these problems exist. We know that they limit our interpretations of past activities. We need to approach these problems more systematically at a fundamental level.

We need to find out more about what exactly is causing external particles to become trapped inside our dental calculus, and be able to quantify exactly how they impact our interpretations of dietary practices from archaeological dental calculus.

My dissertation introduces a potential method for resolving these issues, namely a protocol for growing artificial dental calculus in a lab. Working with a very controlled model of dental calculus in a lab allows me to explore the influence of a wide range of factors that may influence the uptake of particles into dental calculus, and what biases are introduced by these factors as well as the methods we currently use to extract information from archaeological dental calculus. Addressing these fundamental issues and limitations will go a long way towards improving the resolution of our interpretations of past dietary activities. I also explore new ways to extract information from archaeological dental calculus to learn more about our past.

Chapter 1 is a brief introduction into the many uses of archaeological dental calculus to reconstruct the diet of past populations. I also outline the current state of dental calculus research, and some of the problems we are facing. I only briefly describe what dental calculus is, and how it is formed; this is an important concept to understand, since it influences the uptake of food particles, and is influenced by diet. **Chapter 2** provides more detail on the formation and growth of dental plaque, and mineralisation to form dental calculus. Here, I also provide an overview of oral biofilm models to provide some context for the experiments I conducted in my dissertation research using an oral biofilm model

Chapter 3 is the first article, which introduces the oral biofilm model I developed for my research. In this article we also assess the ability of our model to mimic the properties of natural dental calculus in order to justify using model calculus as a proxy for archaeological dental calculus. By characterising the

bacteria present in the model calculus, we found that it was indicative of an oral microbiome, though somewhat distinct from the natural calculus we used as a comparison. We also determined the mineral content using Fourier Transform Infrared (FTIR) spectroscopy, which established that the model was primarily made up of carbonate hydroxyapatite, the predominant mineral in natural dental calculus. The second article that forms **Chapter 4** we applied the calculus model to find out what happens when we add a known amount of dietary starch granules to model calculus during formation, and attempt to extract the starches using a common method for extraction of starches from archaeological dental calculus. We were able to validate what previous studies on modern humans and non-human primates have shown, that the quantity of starches that we extract from calculus, is not very representative of the dietary intake. We also discovered that one of the causes for this misrepresentation of the starch record is that large starch granules were being incorporated at a lower rate than smaller granules. This was demonstrated by the fact that potato granules, which are quite a bit larger than wheat, were underrepresented in our extracted counts.

In addition to diving into the causes behind dental calculus limitations, we also sought out to find novel uses for archaeological dental calculus. In **Chapter 5**, the final article in this dissertation, we used a novel method employing ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to identify various plant-based alkaloids traditionally used for medication and non-dietary activities, such as nicotine, opioids, and cannabinoids. We were unable to detect opioids and cannabinoids, but we found evidence of alkaloids and metabolites derived from the consumption of tea and/or coffee, as well as nicotine (and its metabolite, cotinine), and salicylic acid, the primary phytohormone in willow bark. We combined these results with the presence of skeletal and dental indicators of disease to find patterns of potential disease management. We were unable to definitively link the presence of these with evidence of disease to justify medicinal activities, but we found some interesting correlations between maxillary sinusitis and markers of tobacco-use and consumption of tea and coffee.

In the final part of the dissertation, **Chapter 6**, I discuss the outcomes of the studies from previous chapters, addressing the oral biofilm model and its implications for archaeological research. I lay out the challenges we need to accept to further our understanding of archaeological dental calculus and how it relates to the dietary activities of the people we study in the past. This includes systematically conducting more fundamental research to understand the mechanisms causing various dietary (and non-dietary) markers to become entrapped in dental calculus.

Samenvatting

Translation by Esther Plomp

Tandsteen. Deze kleine, harde, onopvallende substantie vormt op de tanden van mensen en dieren. Tandsteen bevat verrassend veel informatie over ons leven. Tijdens de vorming en groei is tandsteen een levende biofilm plak en neemt het een grote verscheidenheid aan zeer kleine deeltjes op uit verschillende bronnen, met name bacteriën en voedselresten. Deze bronnen, en de deeltjes die ze achterlaten in onze mond, worden beïnvloed door activiteiten en biologische processen die uniek zijn voor elke persoon, dankzij onze eetgewoonten, mondhygiëne, genetica en de omgeving waarin we leven.

Wat het zo interessant maakt voor archeologen is dat deze deeltjes ingebed zijn in het tandsteen na de mineralisatie van de tandplak. Hierdoor zijn de deeltjes goed beschermd zijn tegen verwijdering en afbraak gedurende honderden tot duizenden jaren in de bodem, waardoor zij een beeld kunnen geven van de activiteiten van de mens. Archeologen kunnen dit beeld ontrafelen door eiwitten van planten en dieren, evenals genetisch materiaal en microresten van planten die gevangen zitten in de kalkmatrix, te extraheren en te identificeren.

Het grote probleem - of één van de grote problemen, want er zijn meerdere - is dat dit beeld nooit een compleet beeld is van een leven vol activiteiten. Een ander probleem is dat het beeld in de loop der jaren vervaagd en dat sommige

delen volledig uitgewist zijn. Er zijn veel dingen van invloed op wat ingebed raakt in tandsteen, wat er al die jaren bewaard blijft, en hoe het tandsteen geanalyseerd kan worden. Dit heeft invloed op de hoeveelheid informatie die we uit tandsteen kunnen verkrijgen en op de manier waarop we deze informatie interpreteren.

We weten dat deze problemen bestaan. We weten dat ze onze interpretaties van activiteiten in het verleden beïnvloeden. Het is noodzakelijk om deze problemen op een meer systematische wijze op een fundamenteel niveau aan te pakken. Hoe raken externe deeltjes precies vast in ons tandsteen? Ook moeten we preciezer kunnen kwantificeren welke factoren onze interpretaties van voedingsgewoontes beïnvloeden.

Mijn proefschrift introduceert een potentiële methode om deze problemen op te lossen. Deze methode bestaat uit een protocol voor het kweken van kunstmatig tandsteen in een laboratorium (model tandsteen). Door te werken met een gecontroleerd model van tandsteen in een laboratorium kunnen een breed scala aan invloeden op tandsteen onderzocht worden. Bijvoorbeeld de factoren die van invloed zijn op de opname van deeltjes in tandsteen, welke vertekeningen hierdoor worden geïntroduceerd, evenals de invloed van methoden die we momenteel gebruiken om informatie te halen uit archeologische tandsteen.

Het aanpakken van deze fundamentele problemen en beperkingen zal de resolutie van onze interpretaties van eetgewoonten in het verleden verbeteren. Ik onderzoek ook nieuwe manieren om informatie uit archeologische tandsteen te onttrekken om zo meer te weten te komen over ons verleden.

Hoofdstuk 1 is een korte introductie over de vele toepassingen van archeologische tandsteen om het dieet van vroegere populaties te reconstrueren. Ook schets ik de huidige stand van het tandsteenonderzoek, onder andere enkele de problemen waarmee we worden geconfronteerd. Ik beschrijf slechts kort wat tandsteen is en hoe het wordt gevormd. De vorming van tandsteen heeft invloed op de opname van voedseldeeltjes, en wordt tegelijkertijd ook beïnvloed door het dieet. **Hoofdstuk 2** biedt meer details over de vorming en groei van tand-

plak en het mineralisatie proces van tandsteen. Hier geef ik ook een overzicht van orale biofilmmodellen om enige context te bieden voor de experimenten met oraal biofilmmodel die ik heb uitgevoerd in mijn proefschriftonderzoek.

Hoofdstuk 3 is het eerste artikel waarin het orale biofilmmodel wordt geïntroduceerd dat ik voor mijn onderzoek heb ontwikkeld. In dit artikel beoordelen we het vermogen van ons model tandsteen om de eigenschappen van natuurlijke tandsteen na te bootsen. Hierdoor zou het model als proxy kunnen dienen voor archeologisch tandsteen. Door de bacteriën in het model te karakteriseren, vonden we dat het model indicatief was voor het microbioom in de mond (alhoewel enigszins verschillend van natuurlijk tandsteen). We bepaalden ook het mineraalgehalte met behulp van Fourier Transform Infrared (FTIR) spectroscopie, waarmee we vaststelden dat het model voornamelijk bestond uit hydroxyapatietcarbonaat, het overheersende mineraal in natuurlijk tandsteen.

In het tweede artikel dat **Hoofdstuk 4** vormt, pasten we het calculusmodel toe om uit te zoeken wat er gebeurt als we een bekende hoeveelheid zetmeelkorrels toevoegen aan het model tandsteen tijdens de vorming van tandsteen. We probeerden de zetmeel korrels te extraheren met behulp van een gebruikelijke methode voor het extraheren van korrels uit archeologische tandsteen. We waren in staat om te valideren wat eerdere studies op moderne mensen en niet-menselijke primaten al hebben aangetoond, namelijk dat de hoeveelheid zetmeel in tandsteen niet representatief is voor de voedselinname. Dit komt omdat grotere zetmeel korrels langzamer worden opgenomen in vergelijking met kleinere korrels. Zo waren aardappelkorrels, die een stuk groter zijn dan tarwekorrels, ondervertegenwoordigd in onze geëxtraheerde tellingen.

Naast het onderzoeken van de oorzaken achter de beperkingen van tandsteen analyses, hebben we ook gezocht naar nieuwe toepassingen voor archeologische tandsteen. In **Hoofdstuk 5**, het laatste artikel in dit proefschrift, gebruikten we een nieuwe methode met ultrahoge prestatie vloeistofchromatografie-tandem massaspectrometrie (UHPLC-MS/MS) om verschillende alkaloïden op basis van planten te identificeren die traditioneel worden gebruikt voor medi-

catie en activiteiten die niet gerelateerd zijn aan het dieet (zoals nicotine, opioïden en cannabinoïden). We konden opioïden en cannabinoïden niet detecteren, maar we vonden wel bewijs van alkaloïden en metabolieten afkomstig van de consumptie van thee en/of koffie, evenals nicotine (en de metaboliet daarvan, cotinine), en salicylzuur (het primaire fytohormoon in wilgenbast). We combineerden deze resultaten met de aanwezigheid van skelet- en tandheelkundige indicatoren van ziekte om zo patronen te vinden van mogelijke ziekte behandelingen. We waren niet in staat om een definitief verband te leggen tussen ziektes en medicinale activiteiten, maar we vonden wel enkele interessante correlaties tussen sinusitis maxillaris en tabaksgebruik en thee- en koffieconsumptie.

In het laatste deel van het proefschrift, **Hoofdstuk 6**, bespreek ik de uitkomsten van de studies uit voorgaande hoofdstukken, waarbij ik inga op het orale biofilmmodel en de implicaties ervan voor archeologisch onderzoek. Ik beschrijf de uitdagingen die we moeten aangaan om ons begrip van archeologische tandsteen te vergroten om zo het dieet van de mens in het verleden beter te kunnen bestuderen. Dit vereist het systematisch uitvoeren van fundamenteler onderzoek om de onderliggende mechanismen beter te begrijpen waardoor verschillende voedingsmarkers (en niet-dieetmarkers) ingebed worden in tandsteen.

Curriculum Vitae

Bjørn Peare Bartholdy, born in the Great White North in a time where we still thought Compact Discs and disposable cameras were pretty neat. I obtained my BSc (honours) in archaeology with a concentration in physical anthropology at the University of Calgary in 2015. My thesis was supervised by Dr. Mary Anne Katzenberg and explored the application of micro-CT in archaeological examination of osteoarthritis lesions in the vertebrae of un-provenanced skeletal remains. I went on to get my MSc in Osteoarchaeology at Leiden University in 2017. Here, my thesis research was supervised by Dr. Andrea Waters-Rist, where I looked for relationships between enamel properties and dental disease in the deciduous dentition, using micro-CT. The materials used for this research were from a 19th century Dutch population.

I started my PhD position in 2017, under the supervision of Dr. Amanda Henry. The main focus of the research involved developing and validating a protocol to develop a model dental calculus system, which could be used to explore dietary research questions from an archaeological perspective. The protocol was validated using Fourier Transform Infrared (FTIR) spectroscopy to assess the mineral composition, and metagenomic analysis to characterise the microbiome. The protocol was then applied to explore questions related to the incorporation and retention of dietary starches in dental calculus.

Alongside my PhD research, I participated in a variety of activities both related

and completely unrelated to my dissertation research. I helped develop and co-instructed the Quantitative Methods in Archaeology course for two consecutive years. I co-supervised multiple Master's students in the MSc Osteoarchaeology specialisation. I also taught a number of guest lectures on dental calculus, dental diseases, statistics, and open science. I was a teaching assistant in the Osteoarchaeology course for third year Bachelor's. I received my Data Carpentry teaching certification and co-instructed multiple Data Carpentry workshops teaching the R Statistical Programming language to social scientists, co-hosted by Leiden University, TU Delft, Erasmus University Rotterdam, and Vrije Universiteit Amsterdam. In 2022 I started as a Data Steward at TU Delft. I've done lots of other stuff, too, which you can find in the CV I can actually keep updated.