Putting dental calculus under the microscope

Bartholdy, B.P.

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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