A Mineralized Frame of the Human Past: A study of in vitro dental calculus for assessing its potential in archaeological diet reconstruction

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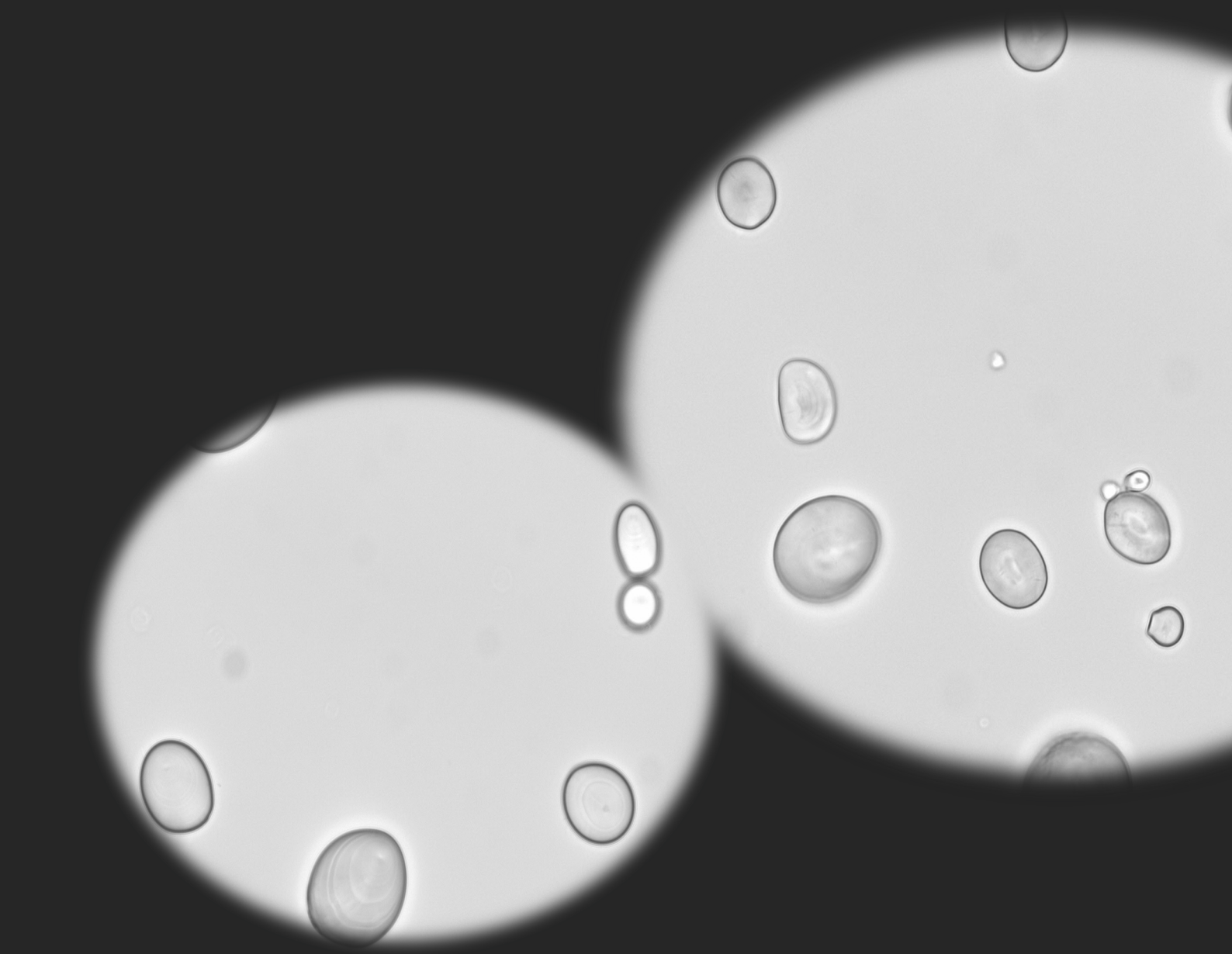

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Cover picture:

Wheat starch granules (right) and potato starch granules (left) seen through the optical microscope. Pictures taken by the author.

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Master Thesis

Table of contents

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1. Introduction

Dental diseases and dental calculus are a peculiarity that has been found in human remains from every historical context (Smart, 1978), and have proved to be traceable to the first stages of human prehistory (Hardy et al., 2012). Hardy and colleagues (2016), indeed, were able to gain insights into diet and environment from dental calculus recovered in a 1.2 million years old man in Spain. Nonetheless, there is no doubt that the frequency of dental disease has significantly increased when human populations moved from being hunter-gatherers, to adopting a subsistence more based on agriculture and animal husbandry around 8,000 BC (Eshed et al., 2007). It has been demonstrated that a diet with large consumption of abrasive substances (Smith, 1972) and rich in sugar, like the one that characterizes the first Neolithic human groups, results in an increase in dental diseases (Eshed et al., 2007). Furthermore, it has been proved that, because of a radical change in diet, poor oral health could also be somehow related to cardiovascular disease (González Navarro et al., 2017). Oral diseases are thus assessed to be one of the main responses to subsistence patterns (Turner II, 1979), and for this reason, they are employed by anthropologists and archaeologists with the aim of analysing the dietary habits of past populations (Hillson, 1996).

In light of these patterns, in the last fifteen years, it has been of increasing importance for osteoarchaeologists to investigate the nature of dental diseases and their relationship with the surrounding environment (Marklein et al., 2019). In fact, by analysing this aspect it would be possible to gain insights into the habits and behaviour of past populations in relation to their living context. Within this framework, the analysis of human remains from archaeological contexts has provided a good means for hypothesis testing and, in addition, the support that archaeological research on dental diseases offers to modern health studies has been documented (Whittaker, 1990).

1.1 Dental calculus in archaeology

Teeth are one of the most commonly found human remains in archaeology and they show great preservation in most taphonomic contexts (Kendall et al., 2018). This characteristic is due to the hard component that constitutes the enamel, hydroxyapatite, and makes the dentition the "hardest" part of the body (Scott & Turner, 1997). The core of the tooth is comprised of the pulp chamber, a hollowed-out space in which blood vessels and nerves supply nourishment (Riquieri, 2019). To cover this inner area is the dentin, a calcified connective tissue that runs from the crown, the external portion, to a socket in the jaw in which the tooth is set. The dentin itself is covered by enamel, which is hard and brittle and is the only tooth component that is in contact with the external environment (White & Folkens, 2005). Below the gum line, the enamel is not present, and the roots of the teeth are covered by a calcified substance called cementum (Figure 1. 1)

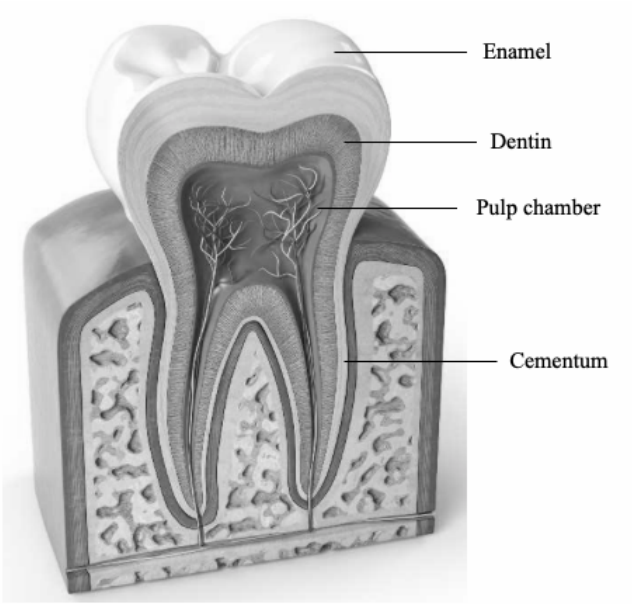

Figure 1. 1: Anatomy of a human tooth (Sezen et al., 2018, p. 18)

Throughout the life of an individual, a complex structure of bacteria and other microorganisms known as dental plaque accumulates on the enamel of each tooth (Rosan & Lamont, 2000). Eventually, by means of a process that will be explained in detail in the next chapters, the dental plaque mineralises and results in the formation of dental

calculus. Based on the location in which this occurs, dental calculus is deemed to have slightly different characteristics in bacterial and mineral components (Radini et al., 2017), as well as variation in volume (Dobney & Brothwell, 1987). The two different areas in which the calculus forms are the supragingival portion, above the gum line, and the subgingival section, below the gingiva (Akcalı & Lang, 2018).

As previously mentioned, dental calculus from archaeological contexts is now broadly studied. The increasing interest in this human material is due to its propensity to mineralise fragments of materials that somehow enter the oral cavity, within its matrix. Actually, thanks to the analysis of dental calculus, plant microfossils such as starch granules (Hardy et al., 2009), phytoliths (Leonard et al., 2015), charcoal (Wesolowski et al., 2010) and cotton fibres (Blatt et al., 2011) have been recovered and have helped archaeologists and physical anthropologists to reconstruct not only the dietary patterns but also the daily habits (Blatt et al., 2011), such as the use of the mouth as a third hand and specific activities (Lustmann et al., 1976). Finally, more recently calculus has been used as a mine to extract bacteria and microorganisms, which, thanks to their DNA, can help researchers to investigate human health over time (Weyrich et al., 2015).

Although several studies have been carried out on archaeological samples, it would be interesting to obtain comparisons from modern populations. The study and observation of present-day material would indeed allow manipulation and control of the environment, and thus it could help to get clearer insights into calculus formation and the role of food in this process. When dealing with archaeological dental calculus it is not possible to recreate these conditions. However, working with human subjects is challenging and ethically complex, especially for studies that would require detailed dietary recording over the long time periods required for calculus formation, and thus very little research has been conducted. *In vitro* analysis of dental calculus sets itself as a realistic approach to solve these ethical issues (Tatevossian, 1988). The creation of an “artificial mouth” (Hudson et al., 1986) enables researchers to control the experimental conditions and to manipulate the experiment itself (Sissons et al., 1991) in order to obtain clear and unbiased results. Despite these hopeful premises, barely anyone has approached this methodology with the purpose of better understanding the analysis of archaeological dental calculus. Almost nothing is known about the whole process by which different types of food fragments become trapped within the dental calculus and it would be relevant to assess this step before carrying out an analysis whose results could be

erroneous and biased by incorrect knowledge.

This study will focus on dental calculus, and particularly on the dietary information that this material is able to preserve throughout time. The potential of calculus to enable the reconstruction of food intake of past populations has been demonstrated by several studies (Hardy et al., 2018; Mickleburgh & Pagan-Jimenez, 2012; Power et al., 2018) and new important aspects have been considered. A crucial factor of dental calculus analysis, however, is the poor knowledge of the process that leads to its formation and the action that results in trapping food debris (Lieverse, 1999). It is not clear to what extent the differences between people's oral environments can encourage or restrict dental calculus development (Lieverse, 1999), and, on the other hand, it has not yet been understood which are the mechanisms which lead to the embedding of food particles within the calculus matrix.

1.1.1 Potential and limitations for diet reconstruction

The first investigation of dental calculus can be attributed to Armitage (1975) who performed the extraction of food debris from animal mineralised plaque, followed by Dobney & Brothwell (1986) that carried out this same procedure in human dental calculus from archaeological samples. Thanks to these studies it was possible to evaluate the state of preservation of debris within the calculus matrix and assess the potential of this material to conserve intact evidence of the human past (Fox et al., 1996). Starting from these analyses, several investigations have followed with the aim of exploring the dietary patterns of our ancestors. The analysis of sediments and tools to gain information on past nutrition is indeed likely to contain biases or be incomplete due to misleading data (Li et al., 2010). The debris found trapped in dental calculus have been considered traces of food intake, human habits (Gismondi et al., 2018) and only recently, evidence of the environment surrounding the individual (Radini et al., 2017).

The archaeological research of dietary evidence is particularly supported by the extraction of particles such as phytoliths and starch granules from dental calculus (Wesolowski et al., 2010). Nonetheless, the recovery of starch granules in mineralised human dental plaque does not simply reflect the consumption of food and, at the same time, plant intake does not necessarily result in preserved debris in dental calculus (Mickleburgh & Pagán-Jiménez, 2012). As a consequence, predicting the amount and frequency of starchy food

ingestion according to the number of recovered debris is almost erratic and unreliable (Mickleburgh & Pagán-Jiménez, 2012). Furthermore, starch granules are highly prone to undergo taphonomic modification. In particular, the effect of water, heat and enzymes result in a morphological change in the grains themself that can make their identification challenging (Henry, 2014). Especially the process of gelatinization caused by the heating of the starch granules and the breaking of the bonds in the molecules due to the action of digestive enzymes create distinctive patterns on their surface (García-Granero, 2020). However, it is equally possible that a strong action of these agents might cause the complete degradation of starchy particles and thus keep them from being preserved in dental calculus and archaeological contexts (Copeland & Hardy, 2018). The processes that lead to the starch granules deposition and preservation in dental calculus are strictly linked to the formation of the calculus itself, the nature of the food molecules and the action of the depositional processes (Hillson, 1996). As a consequence, it is undeniable that the analysis of food debris extracted from dental calculus and the information inferred from it are strongly affected by the surrounding context and all the possible scenarios need to be considered.

1.2 Research questions

Previous analysis on dental calculus has focused on archaeological specimens with the purpose of inferring new insights about the dietary habits of past populations (Radini et al., 2017). Debris such as phytoliths, starch granules, charcoal and cotton fibres (Blatt et al., 2011) have been extracted from the calculus matrix over the years, and these materials have helped the researchers to speculate on the paleoenvironment (Asevedo et al., 2020), trying to reconstruct the surroundings in which the humans of the past lived.

Nevertheless, the process of dental calculus formation and, particularly, the way in which the various debris get trapped within the matrix is not fully understood (Lieverse, 1999). The consequence of investigations carried out using a material that has not yet been entirely comprehended can result in biased interpretations. Before proceeding with archaeological studies and inferring past behaviours using dental calculus analysis, it would be advisable to ensure the right interpretation of the data by a deep understanding of the examined material.

In this framework, this study aims to assess how different types of starch granules get incorporated within the dental calculus matrix throughout its formation in the oral environment. To address these issues, an *in vitro* oral biofilm was created starting from artificial and whole saliva collected by two donors; the solution was eventually exposed to starch granules and mineralized to obtain an accurate reproduction of dental calculus. While *in vivo* studies with human subjects might be preferable, this is difficult due to the variability that characterized the human saliva (Shellis, 1978) and the ethic that surrounds the topic (Radini et al., 2017). In order to create a condition as similar as possible to the real circumstances in which dental calculus formation takes place, I added to some of the samples alpha-amylase, an enzyme present in human saliva with the function of starch degradation. Furthermore, starch granules of two different taxa and distinct processings have been added during the calculus formation protocol to analyze the different responses.

The research, therefore, aims to answer the following main question:

- How well are dietary habits represented by starch granules extracted from dental calculus?

Furthermore, in order to accurately address this enquiry, the following sub-questions were raised:

- To what extent is the effective quantity of starch granules entering the oral cavity still visible in the dental calculus after the mineralization of the dental plaque?
- How does alpha-amylase influence the starch count in dental calculus?
- How does the difference in the structure of processed and native starch granules affect the intake of the granules themselves within the oral biofilm?

Limited research has been carried out on the process that leads the debris to the incorporation of debris within the mineralized plaque, and this represents the major limitation in archaeological studies on dental calculus (Lieverse, 1999). The benefits of a better understanding of this scheme can bring advantages not only to the archaeological field but also to the medical environment. Thus, further investigations on the topic are desirable, with the purpose of ultimately defining with certainty the relationship between food intake and the mineralization of the debris. This would help to reduce the bias in the interpretation of the data and it would lead to more transparent research.

1.3 Thesis structure

The thesis will be divided into 6 chapters. The first and current one is a brief introduction to the topic, with an outline of the status of the research and the characterization of the problems related to the study of dental calculus. The aim of this study is then described as a strategy for overcoming the limitations of dental calculus analysis. In Chapter 2, I will explain in detail the different elements that constitute a crucial part of this research, so that the reader could become familiar with them. In addition, a historical overview of dental calculus studies will be presented. Chapter 3 will show the protocols and methodologies that were used throughout the research for both the *in vitro* formation of dental calculus and the further analysis carried out with the material produced. The data obtained from the investigation and analysis will be presented in Chapter 4 and they will be then discussed in Chapter 5. Finally, Chapter 6 will draw the conclusions of the investigation trying to give an answer to the research questions previously outlined and exploring the limitations and the future possible developments that this study brings.

2. Background

Dental calculus is considered to be one of the most evident widespread dental condition in both ancient and modern populations (Adler et al., 2013). When analyzing the archaeological human remains, it is very frequent to face this hard formation still on the teeth because of its particular properties. A proof of its tendency to preserve over a long period of time is the discovery of a maxilla dated to the late Pliocene with fragments of calculus still attached on the surface of the teeth (Blumenschine et al., 2003). It is only in the last fifteen years that scientists discovered the importance of dental calculus in archaeological research and since that moment its potential has been exploited, obtaining relevant results on various topics, ranging from the dietary investigation, as described in the research of Buckley and colleagues (2014), to DNA analysis (Preus et al., 2011).

This chapter will provide a historical overview of the dental calculus analysis and a description of every component that has constituted a key role in this research. In the beginning, a summary of the historical background will be presented. As a second point, the biological construction and aetiology of dental calculus will be extensively described, and its potential for archaeological research will be outlined, emphasizing the prospective for the investigation of diet reconstruction. Thirdly, since the experiment focused on the count of starch granules, a description of these molecules will be provided, as well as the possible modification of their morphology in contact with different external factors. The fourth and last topic, alpha-amylase, the enzyme used throughout the experiment for recreating an environment as similar as possible as the real oral one, will be briefly explained.

2.1 Historical overview

The first to ever approach dental calculus as a source of information about past diets was Armitage (1975) who analyzed phytoliths from calculus on the surface of cattle teeth. The result of this investigation led to the conclusion that dental calculus had the potential for diet reconstruction in ancient environments. Eventually, Dobney and Brothwell

(1986) applied this analysis to calcified plaque recovered on human remains, assessing the good state of preservation of the food debris found within it. They were able to identify fragments such as cereals, pollen grains and phytoliths. The possibility of using dental calculus for the investigation of the oral microbiota was proved by the same author (1988) using scanning electron microscopy (SEM) (Wright et al., 2021); they were indeed able to detect bacteria mineralized within the calculus of Neanderthal. Dobney and Brothwell also developed a method for evaluating the amount of dental calculus recovered on tooth surfaces, enabling the archaeologists to have a standardized method to use during the analyses. The protocol consists of giving a numerical score to the thickness of the calculus from 1 to 3, where 1 is the lowest and 3 is the highest (Brothwell, 1981).

Starting from this period the investigation for both dietary reconstruction and DNA analysis have followed one another. It is important to cite the remarkable works carried out by Ciochon and colleagues (1990), in which they were able to identify phytoliths within the dental calculus matrix of *Gigantopithecus blacki*, an extinct ape, and the research of Capasso, Di Tota, Jones and Tuniz (1995), where they assessed the potential of synchrotron radiation microprobe for analysis of trace elements in the calculus matrix. In the last fifteen years, the studies on mineralized plaque have increased broadly, mostly focusing on the identification of the debris entrapped within the calculus, and the extraction of DNA for microbiome investigations.

2.2 Dental calculus

Dental calculus is the result of the mineralization of the dental plaque which accumulates overtime on the surfaces of the teeth (Jepsen et al., 2011; Lieverse, 1999). There are two different types of dental calculus; supragingival, which forms above the gingiva, and subgingival, which instead occurs below the gingiva (Akcalı & Lang, 2018; Schroeder, 1969). Supragingival calculus appears to be more present on the lingual surfaces of anterior teeth in the mandible and on the buccal surface of the third molars in the maxilla (Parfitt, 1960); these locations are also characterized by the occurrence of salivary ducts (Jin & Yip, 2002). On the other hand, subgingival calculus seems to be more spread on the lingual surfaces rather than the buccal, particularly on the lower first molars (Jin & Yip, 2002). In addition, it is important to note that while the individual is still alive, the dental calculus is perpetually covered with a thin layer of non-mineralized plaque (Jepsen et al., 2011). The result of this process is the build up of numerous dental

calculus layers and the capacity of the oral biofilm to constantly embed the food particles throughout the life of an individual.

The formation of dental calculus always begins with the deposition of the dental pellicle, a thin organic layer positioned on the tooth's surface; this process is attributed to the activity of saliva or crevice fluid (Lieverse, 1999). Immediately after, this biofilm is colonized by bacteria and various other organisms that find nourishment from the components of saliva (Mandel, 1987). It has been estimated that around 100 billion bacteria are present per gram of dental plaque (Charlier et al., 2010) and scientists count over 700 different bacteria species (Mackie et al., 2017). It has been repeatedly stated that the presence of these bacteria is not fundamental in the constitution of the dental calculus itself (Fitzgerald & McDaniel, 1960) but that however, their permanence in the initial biofilm facilitates the formation of calculus in humans.

Plaque mineralization occurs when calcium phosphate minerals are deposited on the biofilm colonized by microorganisms and occurs through the bond of calcium ions with carbohydrate-protein complexes that derive from the organic part (Mandel, 1960). This process is believed to be caused by saliva for the supragingival calculus, and by the gingival crevicular fluids for the subgingival calculus (Jepsen et al., 2011). The calcification process begins as early as the second day (Akcalı & Lang, 2018), but it is considered complete around the twelfth day (Muehlemann & Schroeder, 1964). Four different types of calcium phosphate crystals make up the inorganic component of the dental calculus; these are brushite, octacalcium phosphate, hydroxyapatite and whitlockite (Schroeder, 1969), with hydroxyapatite being the predominant one.

The development and the formation of the crystals is not random; instead, brushite and octacalcium phosphate form first, while, hydroxyapatite and whitlockite use them as precursor crystals and thus they became abundant only at a later stage (Driessens & Verbeeck, 1989). Dental calculus analyses with SEM and TEM highlight its tendency to mineralize in layers that can reach 20-400 μm (White, 1997) and show that the different layers are interspersed with pellicle-like incremental lines (Schroeder, 1969), suggesting mineralization punctuated by phases of organic consumption. It has been demonstrated that μCT scan enables the investigation of dental calculus internal structure showing clearly the multiple levels of layering and provides a non-destructive technique for calculus analysis (Power et al., 2022). Finally, it is important to note that the speed and pattern with which dental calculus grows is not standardized and varies strongly from

person to person (Jepsen, 2011).

2.2.1 Dental calculus aetiology

Many factors influence the process of mineralization and some of them are still not fully understood. It is believed that variables such as age, gender, diet, bacterial composition and diseases can affect the rate of dental calculus growth and mineralization in a predominant way (White, 1997). Starting from the "Booster Mechanism" elaborated by Mandel (1990), a great number of studies have been carried out on the chemical composition differences that affect the environment in which dental calculus grows, comparing individuals prone to calculus development with people that appear to be less subjected to its formation (Lieverse, 1999). It has been noticed that urea, calcium, proteins and phosphate are present at higher levels in the saliva of people with the tendency of calculus formation compared to the others (Mandel & Thompson, 1967), suggesting the relevance of the calcium and phosphate concentration in the oral environment for determining the inclination of an individual to develop calculus during their life (Mandel, 1974). Nonetheless, the process that leads to the mineralization of dental calculus is much more complex than this. The increase in alkalinity levels seems to simplify the precipitation of calcium and phosphate, and it is also thought that bacteria could be responsible for the rise of the alkaline concentration and for encouraging mineralization through the breakdown of mineralization inhibitors (Scheie, 1989).

Diet is also believed to be a factor that affects the formation and growth of dental calculus. The contribution of diet has been investigated by researchers and most of the time the studies are guilty of oversimplification (Lieverse, 1999). This lack of documentation regarding the relationship between diet and dental calculus in humans is however compensated by large studies applied to the animal world (Schroeder, 1969). Surprisingly, thanks to several research on rats, it has been assessed that calcium and phosphate in food do not impact the calculus formation, and, on the other hand, a diet rich in bicarbonate does it (Kakehashi et al., 1963). Similarly, high fats and high carbohydrate diets affect dental calculus by reducing the speed of its formation (Smith et al., 1963). It has also been demonstrated that individuals with large water intake, as well as high consumption of proteins, appear to be associated with a low dental calculus presence (Hillson, 1979). Indeed, the proteins assumed with food increase the levels of blood urea and since the mineral concentration is related to other fluids, they result in increasing oral

fluid urea levels as well (Dawes, 1970), and thus a diet high in protein indirectly can raise the alkalinity of the saliva. Finally, dental calculus, diet and microorganisms that live in the oral environment are characterized by a strong connection. As previously mentioned, in fact, the dietary habits of an individual can modify the oral microbiota and thus the calculus formation, as well as the presence of dental calculus can lead to the development of dental disease and thus contribute to a general change in the oral bacteria. As the other increases or decreases it is highly probable for changes to occur in the general mouth habitat.

2.2.2 Dental calculus in archaeology and its potential for diet reconstruction

In the last decade in archaeology, dental calculus has gone from being a discarded material that prevented the analyses of dental wear and stress marks, to an irreplaceable resource for the investigation of the human past. There are several reasons why dental calculus nowadays constitutes an essential tool for archaeological research. First of all, the formation and growth of calculus stop at the time of death, and thus, it is very unlikely that micro-debris derived from the environment get trapped in its matrix (Middleton & Rovner, 1994). Second and most important, during the life of the individual, dental calculus traps microorganisms, biomolecules and fragments of various types that come into contact with the oral environment (Figure 2. 1) (Buckley et al., 2014; Radini et al., 2017). Nonetheless, not all debris in the calculus gets mineralized in the matrix as a result of food consumption, in contrast to what may be thought. In fact, there are various ways in which micro fragments in the environment can reach the dental plaque, such as inhalation, the use of the mouth as a third hand, food preparation and secondary eating (Radini et al., 2017), and thus, it is important to considerate this wide range of possibilities when approaching the analysis of archaeological dental calculus.

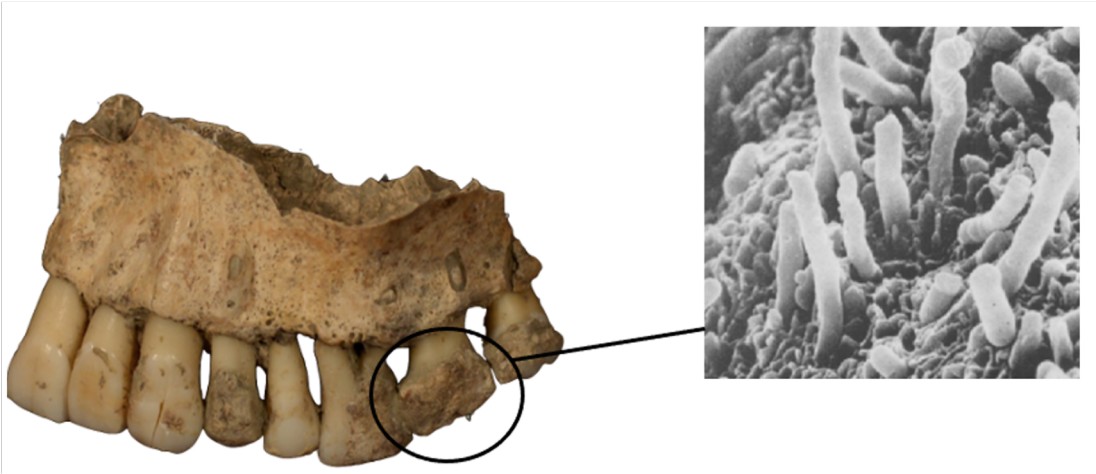

Figure 2. 1: example of dental calculus on maxillar teeth, on the left (picture taken by the author), and the microorganisms projecting from dental calculus under a scanning electron microscope (SEM), on the right (Lustmann et al., 1976, p. 50).

Among osteoarchaeology, it remains controversial whether dental calculus research is truly non-destructive. It is clear that some material must be removed for analysis, however, this is usually a very small amount, and often in situ unsampled material can be left for further investigations (Mackie et al., 2017). Furthermore, dental calculus is not exactly a human tissue, but it is considered to be an ectopic growth, thereby softening the debate over the ethicality of the research. One of the main topics that the investigation of dental calculus has deepened is the research of the human diet over time. In the current archaeological studies, optical microscopy is the most frequent methodology used for the analysis of food debris in calculus to identify dietary components and evaluate the alteration of starch granule (Henry et al., 2011). Furthermore, a wide range of other techniques, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM), is often used in conjunction with microscopy to obtain better recognition (Radini et al., 2017). It is precisely through the identification of particles such as starch granules and phytoliths that it is possible to gain insights into the plant-based diet of people in the past.

2.3 Starch granules: structure and formation

Starch granules are very common carbohydrates composed of two glucose polymers: linear helical amylase and branched amylopectin (Pfister & Zeeman, 2016) and

they represent 98-99% of the dry weight of starch (Tester et al., 2004). Starch-containing plants are widespread and many of them are considered edible. Starch can be found in every tissue of most green plants, such as seeds and leaves (Hardy et al., 2009). In cereals, the starch granules are found in the endosperm, the centre of the grains, while in tubers, starch is the prevalent element and can be found everywhere. Transport and energy storage are the primary tasks that these carbohydrates undertake within the plant (Henry, 2014), and based on their function, starch granules are divided into two different types: transitory and storage starch (Pfister & Zeeman, 2016).

The starch granule starts to grow from the *hilum*, known as the central point of the molecule and its growth develops radially. This process takes place in the amyloplasts within the plant and usually, one starch grows in one amyloplast, though some plants produce multiple starches in each amyloplast (Henry, 2014). The general pattern of growth shows the alternation of crystalline and amorphous layers defined as lamellae (Pérez et al., 2009) that are clearly visible under light microscopy. This pattern is deemed to be characteristic of the different taxa, as well as other features in starch granules such as pores, cracks and vacuoles, facilitating their identification during the analyses. Another aspect that helps the recognition of starch granules is their size and morphology. Usually their size spans from 1μm to 100μm and together with the morphology is strongly peculiar to the different taxa (Hardy et al., 2009).

Starch granules are rather stable molecules, but their structure can be damaged by digestive enzymes (Hardy et al., 2009) or could be subject to modification by water and heat (Henry, 2014). In particular, water can be absorbed by the granules which are able to soak up 0.5 g of liquid every gram of their dry weight (Brown & French, 1977) and, as a consequence, they have an increase of their size, which is visible under the microscope. Likewise, heat creates a remarkable change in the structure of starch granules, giving rise to gelatinization. This process usually occurs above 60° C and it is strongly associated with the level of shear forces that are applied (Hardy et al., 2009) and the amount of water that occurs. The result of gelatinization is the loss of order due to the rupture of the hydrogen bonds between the carbohydrates chains which leads to swelling of the granules and loss of birefringence (Henry, 2014). The starch chains could be prone to return back to an ordered structure, this is known as the process of retrogradation which occurs at the moment in which the temperature of gelatinized starch drops. The result of this process is a stronger starch granule which is more difficult to degrade by the enzymes (Hardy et

al., 2009). In addition, it has been assessed that when observed under a microscope, cooked starch granules are recognized as such, showing a characteristic pattern that is different from starch simply exposed to water (Henry et al., 2009).

Starch granules are considered to be a great resource of energy for several organisms, considering that nowadays foods based on these components provide from 50 to 70% of the total amount of energy in humans (Atkins & Bowler, 2016). Specific enzymes are required in order to break down the starch granules and obtain the energy stored in their crystalline structure. The proteins employed for this process are called amylases. The results obtained from the action of these enzymes can be of two different types and are clearly visible on granules when observed using a microscope. The surface of the starch granules can either be chipped away by the enzymes, assuming a rugged appearance, or they can be eaten away from the inside due to the penetration of the enzymes. This last protein activity results in peculiar openings on the surface of the granules (Galliard & Bowler, 1987; Henry, 2014).

2.4 Alpha-amylase

As stated before, starch-consuming organisms need specific enzymes in order to break down the starch granules and obtain the energy stored in them. There are two amylase groups, called alpha and beta, and they differ in the way they attack the bonds of the starch granules. Alpha-amylase (EC 3.2.1.1) is the most significant amylase in humans, and it has the function of hydrolysing carbohydrate molecules such as starches, into smaller saccharide molecules such as maltose (MacGregor, 1988). Alpha-amylase is most commonly found in pancreatic juice and saliva within the human body. The enzyme found in the saliva is the main responsible for the first digestion of starch granules which takes place in the mouth (Hardy et al., 2009). They are then further broken down in the stomach.

It is important to note that in humans the activity of alpha-amylase varies strongly based on different factors such as stress (Nater et al., 2006) and sleep deprivation (Pajcin et al., 2017) and thus it does not behave in a steady manner. Alpha-amylase is relevant in this context because its presence in the oral environment reduces the number of starch granules by breaking them down. This results in a lower concentration of particles that can be trapped in the dental calculus matrix, and, consequently, in a reduced percentage

of starch granules compared to the one consumed. It is crucial to keep these variables in mind when analysing archaeological material. Finally, the activity of alpha-amylase is expressed by the units of maltose released in a defined volume of the enzyme. This is usually described as U/µL.

2.5 Chapter summary

The purpose of this chapter has been to provide an overview of the topic that will be investigated in this study and put the reader at ease with the main elements that are involved in the research. As the first step, a summary of the history of archaeological dental calculus analysis was outlined, highlighting the progress that led to the current state of knowledge. Following that, a broad description of dental calculus was provided, with a focus on the formation process. The aetiology of this process was then investigated and the limited state of knowledge concerning this topic was brought to light. In addition, the potential of dental calculus for paleodiet studies was described with the aim of presenting the opportunity that this archaeological material offers. In relation to this last matter, the biology and behaviour of starch granules were presented as commonly found debris in dental calculus, and thus as evidence of food consumption. Finally, alpha-amylase was also outlined as an important factor that takes action in the oral environment.

3. Materials and methods

In the previous chapter, a detailed outline of the different components of this research as well as the history and the current state of the knowledge about the dental calculus analysis has been provided. This section will introduce the way in which these elements have been combined to carry out the experiment that led to the formation of the *in vitro* dental calculus itself. In particular, the creation of the oral biofilm will be discussed in detail. A biofilm is a complex structure, a dense micro-community of microorganisms in which different bacteria and cells stick to each other and the surface (Oliveira et al., 2015). The biofilm grows on an inert surface and the organisms can adapt to the environment by modifying their gene patterns (Berger et al., 2018). In this study, an oral biofilm is formed, which involves bacteria and cells characteristic of the mouth environment.

3.1 Oral biofilm growth

In order to create the oral biofilm employed for the investigation, the protocol formulated by Bartholdy & Henry (2021) was followed. This protocol is a modified version of those developed by Sissons and colleagues (1991) and Shellis (1978). The entire protocol was carried out on two polypropylene 24 deepwell PCR plates with a lid containing 24 pegs. It is there that the biofilm growth and mineralization took place during the 25 days of the experiment.

Starting from the whole saliva, contributed by two different donors, the biofilm developed in suspension in artificial saliva within the two plates, and during its formation, the bacteria were constantly fed with sucrose solution for a period of nine days, multiple times a day. At the end of the feeding process, on the 10th day of the experiment, the samples have been immersed in a starch solution several times a day. One plate was exposed to potato starch grains, both native and processed, while the other was exposed to wheat starch granules, both native and cooked.

Starting from the 10th day, alpha-amylase was added to artificial saliva for half of the

wells. In detail, the two plates containing the samples had the set-up described in Table 3. 1 and Table 3. 2. From the 15th day, until the end of the protocol, the oral biofilm was mineralized by dipping the sample into calcium phosphate solution four times a day. During this process, the biofilm was exposed to the starch solutions once a day. At the end of the 24 days experiment, the samples were dried in the incubator and collected for the subsequent analyses.

Table 3. 1: Set up of plate 1.

	1	2	3	4	5	6
A	Proc. potato no amylase	Proc. potato no amylase	Proc. potato no amylase	Proc. potato no amylase	Proc. potato no amylase	Proc. potato no amylase
B	Native potato with amylase	Native potato with amylase	Native potato with amylase	Native potato with amylase	Native potato with amylase	Native potato with amylase
C	Proc. potato with amylase	Proc. potato with amylase	Proc. potato with amylase	Proc. potato with amylase	Proc. potato with amylase	Proc. potato with amylase
D	Native potato no amylase	Native potato no amylase	Native potato no amylase	Native potato no amylase	Native potato no amylase	Native potato no amylase

Table 3. 2: Set up of plate 2.

	1	2	3	4	5	6
A	Proc. wheat no amylase	Proc. wheat no amylase	Proc. wheat no amylase	Proc. wheat no amylase	Proc. wheat no amylase	Proc. wheat no amylase
B	Native wheat with amylase	Native wheat with amylase	Native wheat with amylase	Native wheat with amylase	Native wheat with amylase	Native wheat with amylase
C	Proc. wheat with amylase	Proc. wheat with amylase	Proc. wheat with amylase	Proc. wheat with amylase	Proc. wheat with amylase	Proc. wheat with amylase
D	Native wheat no amylase	Native wheat no amylase	Native wheat no amylase	Native wheat no amylase	Native wheat no amylase	Native wheat no amylase

3.1.1 The artificial saliva

The artificial saliva (AS) used for the experiment was created beforehand following Bartholdy and Henry's protocol (Bartholdy & Henry, 2021). We chose artificial saliva over donated saliva because the latter is highly variable in terms of nutrients and bacterial compositions. In order to create the artificial saliva, as the first step, 300mL of distilled water was added to a beaker and heated to 60°C. Eventually, 2.5g of Mucin from porcine stomach, 5g of Trypticase Peptone, 10g of Oxoid Proteose

Peptone and 5g of Bacto Yeast Extract were added to the warm distilled water and stirred until complete dissolution. The mucin, in particular, has the aim of providing to the artificial saliva the salivary glycoprotein (Shellis, 1978), which is essential for obtaining a solution as close as possible to the real one.

At a later stage, once the reagents were completely dissolved, other chemicals were added to the solution, these were 2.5g of KCl, 0.35g of NaCl, 0.2 g of CaCl2, 0.74g of Sodium phosphate dibasic, 0.54g of NaHCO3 and 2.5mg of Hemin in order to obtain the right alkalinity that would allow bacteria to thrive in it. At this point, the remaining 700mL of distilled water was poured into the beaker and after some stirring, the pH level was tested, with the ideal value of 7. The entire solution was then decanted into two 1000 mL bottles, filling both of them up to half. The two bottles were then autoclaved at 121°C, 1Bar for approximately 50 minutes for the purpose of sterilization. Once the solution was back to room temperature, 1mg of Menadione, 0.3g of Urea and 0.17g of L-Arginine were added to it. In order to obtain a solution of AS with alpha-amylase, the enzyme was included with the other reagents. Namely, for ca. 900mL of AS, 69.58mg of the enzyme was added at the 9th day of the experiment. A new solution of AS without alpha-amylase was made the same day by repeating the same procedure. The final solutions were stored in the fridge at around 4°C and conserved until the end of the experiment.

3.1.2 The CPMU solution

In order to stimulate the mineralization of the oral biofilm, a solution of calcium phosphate monofluorophosphate urea (CPMU) was created. This solution was prepared by adding to 500mL of dH20 0.775g of Calcium Chloride, 0.72g of Sodium Phosphate monobasic, 0.36g of Sodium Fluorophosphate, 0.4g of Magnesium Chloride and 15g of Urea (Wong, 1998). The solution was then stirred until complete dissolution of the reagents and then stored in the fridge at ca. 4°C (Sissons et al., 1991).

3.1.3 The starch solutions

Two different starch solutions were prepared in advance: 0.25% (w/v) starch from potato (Roth 9441.1) and 0.25% (w/v) starch from wheat (Sigma S5127). One container was made of native (unprocessed starches). Additionally, one bottle of starch solution of each plant type was heated to c. 60°C and stirred for approximately 40 minutes until no

more particles were visible, with the aim of obtaining two solutions with processed starch granules. This process took place under a laminar flow hood with positive air-flow and the liquid was prevented from boiling using a thermometer to monitor the temperature. At the end of this process, we obtained four different solutions: two of them with native granules, of both potato and wheat starch, the other two with cooked (processed) granules, one containing potato and the second wheat.

3.1.4 The sucrose solution

The sucrose solution used for feeding the oral bacteria was prepared by adding 25g of sucrose to 500ml of distilled water, thus obtaining a 5% (m/v) solution. The admixture was stirred until the complete dissolution of the sucrose and stored in fridge until the end of the experiment.

3.2 The experiment

At the beginning of the experiment, described in Figure 3. 1, 30ml of whole saliva (WS) from two different donors were collected into two 50ml plastic centrifuge tubes. Both the donors refrained from oral hygiene 24 hours before the collections and consumed no food or drinks other than water in the two hours before in order to avoid contamination of food debris in the donated saliva. In addition, no antibiotics were taken in the last six months. Before the collection, the donors rinsed their mouths with water to clean them and they stimulated the saliva production by chewing a sheet of Parafilm. At this point, the two substances in the tubes were mixed by putting them in one single tube and vortexing the container with the purpose of creating one single solution. Eventually, the whole saliva (WS) was filtered with a sterilized nylon cloth to remove particles and, later on, it was 2-fold diluted in sterile 20% glycerol. The result of this process is 2-fold dilution of whole saliva. The solution obtained was then homogenized by vortexing.

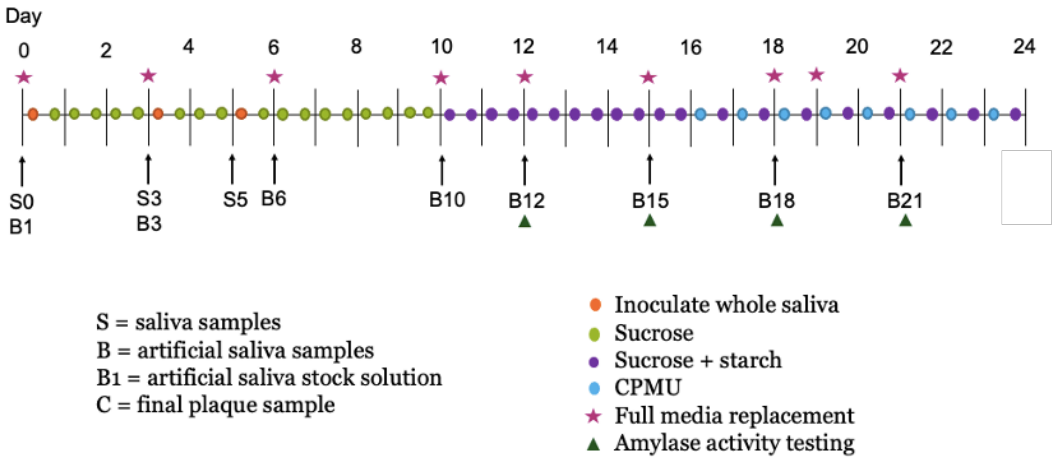

Figure 3. 1: the protocol used for the oral biofilm growth.

After this procedure, 1 ml of WS was inoculated into the wells of two autoclaved 24 deep-well plates, which were then placed for 4 hours in the shaking incubator (Infors HT Ecotron) at 36°C, 30rpm for static inoculation. Once the indicated time had passed, two new autoclaved plates were filled with the artificial saliva (AS); the lids of the plates with the pegs were then transferred into the new plates and kept in the incubator for 4 hours at 36°C. Fresh WS was collected and inoculated as described by the same donors on days 0, 3 and 5 with the aim of providing a good amount of bacteria. In order to feed the bacteria now present in the saliva, from day 0 the samples were transferred to autoclaved plates filled with 1mL of 5% sucrose solution for 6 minutes twice a day; the sugar provides nourishments for the microorganisms present in the oral biofilm and enables them to grow and replicate. On inoculation days the bacteria were fed only once with the sucrose solution.

The sucrose feeding process was replaced by the starch treatment on the 10th day. The starch treatment was carried out by transferring the biofilm into the plates with the starch solution for 6 minutes twice a day and increasing the rpm to 60 to facilitate the contact between starches and the samples. Before every treatment, the plates were agitated allowing the granules to stay in suspension and thus better interact with the biofilm. The starch feeding was carried out using a pipette to avoid cross-contamination between native and processed starch solutions contained in the same plate.

On the same day, the AS contained in the wells was diversified based on the type of treatment; for each plate, two rows, one with native starch granules and the other with

processed ones, were filled with artificial saliva containing alpha-amylase, and the other two rows were pipetted with artificial saliva without alpha-amylase (as seen in Table 3.1 and 3.2). On the 12th, 15th, 18th and 21st days, samples of the AS were collected from the plates with the aim of assessing how the activity of alpha-amylase reacts to the bacteria present in the biofilm. The amylase activity testing was performed at the end of the experiment.

The mineralization of the oral biofilm, and thus the formation of artificial dental calculus, started on the 16th day by submerging the samples in calcium phosphate monofluorophosphate urea (CPMU) solution four times a day for 6 minutes. The mineralization treatment was carried out four times a day until the end of the experiment. In addition to this process, during these days the starch treatment was performed once per day to ensure constant exposition of the oral biofilm to the starch granules. At the end of the experiment, the *in vitro* dental calculus that formed on the pegs was removed from the AS and allowed to dry in the incubator for a night. Once completely dried out, the calculus was collected for further analysis.

3.3 Amylase activity testing

Before the experiment, alpha-amylase activity in the artificial saliva was tested in order to assess whether the enzyme was prone to degradation over time, and to make sure that the activity level was close to that in a human. In order to obtain these results, the enzyme was tested three times over a period of two weeks and compared with whole saliva collected from one of the two donors. As previously mentioned, alpha-amylase strongly shows a great inter- and inter-individual variation, and for this reason we did not expect the activity of the enzyme in the artificial saliva to be identical to the one in the whole saliva; a close value was enough to assess the activity of the alpha-amylase which was used during the experiment.

Throughout the biofilm growth process, samples of AS were collected from the wells after 2 or 3 days of contact with the bacteria and starch granules, with the aim of testing them for alpha-amylase activity. The samples were stored at 4°C in the fridge until day 24 when the test has been performed. In order to conduct the analysis, the AS samples were compared to a standard curve created with 0.2% maltose, distilled water (dH2O) and 75µL of colour reagent created by adding to 12mL of distilled water 8mL of warm

5.3 Molarity (M) Potassium sodium tartrate tetrahydrate and 20mL of warm 96 mM 3,5-Dinitrosalicylic acid solution in an amber bottle (according to the protocol in Bartholdy & Henry, 2021). The different reagents used for the amylase activity testing were added to a plate and portioned so that every well contains 225µL. Both the samples and the standard curves were run in duplicates and both potato and wheat starch solutions were analysed on two different plates. The alpha-amylase activity was detected by filling the wells with 75µL of AS sample and 75 µL of starch solution; the plates were then kept in the incubator for three minutes at 36°C. After three minutes, the plates were removed from the incubator and filled with 75µL of colour reagent. Right after this procedure, the plates were placed in boiling water for five minutes, and afterwards, they were cooled down in ice and diluted with 675µL of water to make the colour variation more visible. The solution was then homogenized using a pipette and 200µL of it from each well was then transferred from the original plate to a 96 well microplate suitable for the microplate reader. Two photometric readings were carried out for each plate in order to avoid computation errors. The microplate reader used for the analysis is the Multiskan FC Microplate Photometer (Thermo Scientific 51119000) with a 540nm filter.

3.4 Sample collection and analysis

The collection of the dental calculus from the pegs (Figure 3. 2) was preceded by the decontamination of the laminar flow hood so that the analysis could run in a safe environment. A selected area of the surface of the equipment was wiped with Kim wipes soaked in dH2O using sterilized tweezers. The cloth was then steeped in 20 ml of dH2O contained in a plastic tube and eventually wrung out in the tube itself. The dH2O was stored for further analysis to assess the degree of contamination before the cleaning process. The laminar flow hood was subsequently entirely cleaned with water and soap and the walls have been wiped with NaOH for sterilization. Finally, dH2O was employed for cleaning the surfaces from NaOH. After the complete decontamination of the laminar flow hood, a chosen area of the working surface was wiped with Kim Wipes soaked in dH2O and the tissue used for it was immersed in a new plastic tube containing 20mL of dH2O. As before the cleaning process, the cloth was then wrung out, discarded and the water stored for further microscope analysis to evaluate the presence of starch granules after the decontamination.

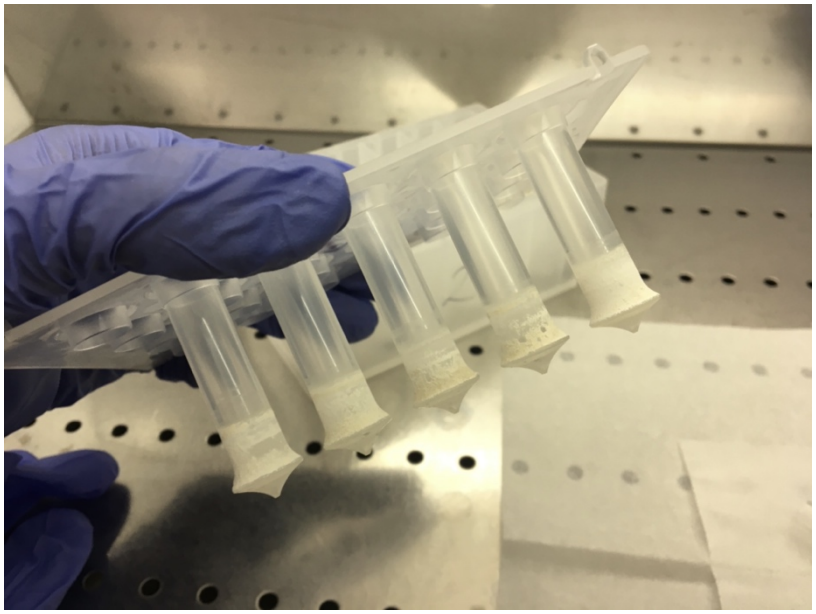

Figure 3. 2: Dental plaque mineralized on pegs at the end of the experiment (picture taken by the author).

In order to collect the mineralized biofilm, a sterilized dental probe was used. The surface of the pegs was scraped to remove the material which was collected on weighing paper lying on two Kim wipes in order to avoid loss of the sample. The dental probe was replaced with a new sterilized one for every row of samples and a different weighing paper was used each time to avoid cross-contamination. The dental calculus removed from the pegs was collected into pre-weighed 2mL conical microtubes; every tube stores one sample and each of them was labelled with the number of the plate followed by the letter of the row and the number of the column. Each microtube was then weighed again in order to obtain the total mass of every sample. In addition, with the aim of assessing how the starch granules behaved to heat and water, the starch solution used for the inoculation throughout the experiment was observed under the microscope. 10µL of each solution were placed on the slides with 10µL of diluted glycerol and covered with an 18x18 coverslip in order to determine the state of the starch granules. Finally, by counting the molecules on the slides it was possible to extrapolate the total number of granules to which the biofilm was exposed and eventually determine the percentage in which the starches were included in the mineralized plaque.

3.4.1 Extraction method

The starch grains were extracted from the calculus by completely submerging the samples contained in the microtubes in 0.5M ethylenediaminetetraacetic acid (EDTA) (Tromp et al., 2017). The samples were then positioned on a vortex shaker for three days until the complete dissolution of the dental calculus matrix. At the end of this process, on a surface cleaned with sodium hydroxide (NaOH), the sample was homogenized using a pipette in order to obtain a solution representative of the whole dental calculus. In order to analyze the content of the dental calculus matrix, 20µL of the dissolved samples in EDTA was placed on a slide covered with an 18x18 coverslip.

3.4.2 The microscopic analysis

The microscope analysis was carried out under a light microscope (Zeiss Azioscope A1) using a 40x objective multiplied by the 10x magnification of the eyepiece. The procedure consisted in counting the number of potato and wheat starch granules that appeared on each sample. In order to make the investigation easier, not all the starch granules present on the slides were counted, but the slide itself was partitioned into three transects representative of the whole slide. Furthermore, the starch granules were divided into three categories based on their size: small (< 10µm), medium (10-20µm), and large (> 20µm), in order to obtain more insights into the starch response to heat and enzyme. In addition, pictures of the starches appearance through the microscope were taken with the aim of providing a visual representation of what has been observed and described. Before counting the starch granules, the dH2O from the pre-sterilization of the laminar flow hood and the post-sterilization were analyzed in order to understand how contaminated was the surface of the machine before and after the cleaning process.

3.5 Chapter summary

In this chapter, the procedures which led to the formation of the dental calculus have been described, highlighting the various steps that enabled the development of the biofilm and the inclusion of starch granules within its matrix. The components outlined in Chapter 2 have been here put together with the final aim of creating a detailed description of the whole process employed in this study. In addition, the reader has been given the opportunity to familiarize themselves with the different elements of the

experiments, and the structure of the samples. Eventually, the methods used to collect dental calculus from the pegs have been explained, as well as the steps that enabled the extraction of starch granules from the calculus and their analysis through the light microscope. Finally, the approach used for the microscopic analysis has been provided and it will be further explored in the following chapter.

4. Results

This chapter will analyse the results obtained from the alpha-amylase assay, the implications of sample weights, and the counts of starch granules within the *in vitro* dental calculus. At first, the results obtained from the amylase activity testing performed on the artificial saliva samples will be converted. The absorbance values detected by the photometer will be translated into U/mL enzyme and described, with the aim of evaluating how the alpha-amylase acts upon different starch granules. As a second step, the mean weight of the dental calculus will be analysed by grouping the samples based on the presence or absence of the enzyme and the process that starch granules undergo prior to their inoculation into the biofilm. This will allow an investigation of the relationship between these two variables and the final dry weight of the mineralized plaque. The total number of granules to which the samples have been exposed will be extrapolated in order to provide the percentage of debris that got trapped within the matrix during the experiment, highlighting the different results obtained on the basis of the size of the granules. Finally, the influence that the presence of alpha-amylase had on the biofilm will be provided by comparing the starch counts obtained from the different samples.

4.1 Alpha-amylase activity detection

The results obtained by the photometric readings show the degrees of light absorbance detected from each of the samples with a wavelength of 540nm. In order to extrapolate the alpha-amylase activity present in the two plates throughout the experiment, several formulae have been run using the values obtained from the standard curve and the blank samples. The steps carried out for this estimation can be summarised as follow:

1) Calculate the ΔA_{540} of each standard by subtracting the standard blank value:

$$\Delta A_{540\ Standard} = \Delta A_{540\ Standard} - \Delta A_{540\ Standard\ Blank}$$

2) Calculate ΔA_{540} of each sample by subtracting the sample blank value:

$$\Delta A_{540\ Sample} = \Delta A_{540\ Sample} - \Delta A_{540\ Sample\ Blank}$$

3) Calculate the mg of Maltose released using the regression coefficients, where y is the $\Delta A_{540\ Sample}$, b is the intercept and a is the slope:

$$\frac{y - b}{a} = mg\ Maltose\ released$$

4) Finally, calculate the units per mL of enzyme:

$$\frac{U}{mL}\ enzyme = \frac{mg\ Maltose\ released}{mL\ enzyme}$$

Thanks to the detection of the alpha-amylase activity in the samples, it is possible to assess how the enzyme acted when added to the oral biofilm. In fact, as we previously verified, alpha-amylase does not degrade over time in the artificial saliva solution (see Section 3.3), however, it has shown to strongly interact with bacteria (Nikitkova et al., 2013).

The artificial saliva samples used for the analysis refer to four different days throughout the experiment and represent every difference in starch type and alpha-amylase presence of both the plates. It is important to point out that the specimens of days 12th, 15th and 18th were retrieved three days after the artificial saliva was refreshed, while the sample on day 21st was collected only two days after fresh artificial saliva was added into the wells.

The calculated values of the alpha-amylase activity are here converted and expressed in U/µL and represent the units of maltose released per mL of enzyme, thus showing how much of the alpha-amylase first added to the original artificial saliva solution, is still acting in the plates after the interaction with the bacteria.

A clear pattern is recognizable from the detection of the alpha-amylase (Figure 4. 1); whether or not the enzyme was inoculated, the results show no or very low activity in each of the samples collected on days 12th, 15th and 18th (see appendix A). On the other hand, the samples exposed to the enzyme, collected on day 21st, reveal values that are close to those obtained for the artificial saliva solution in which alpha-amylase was added.

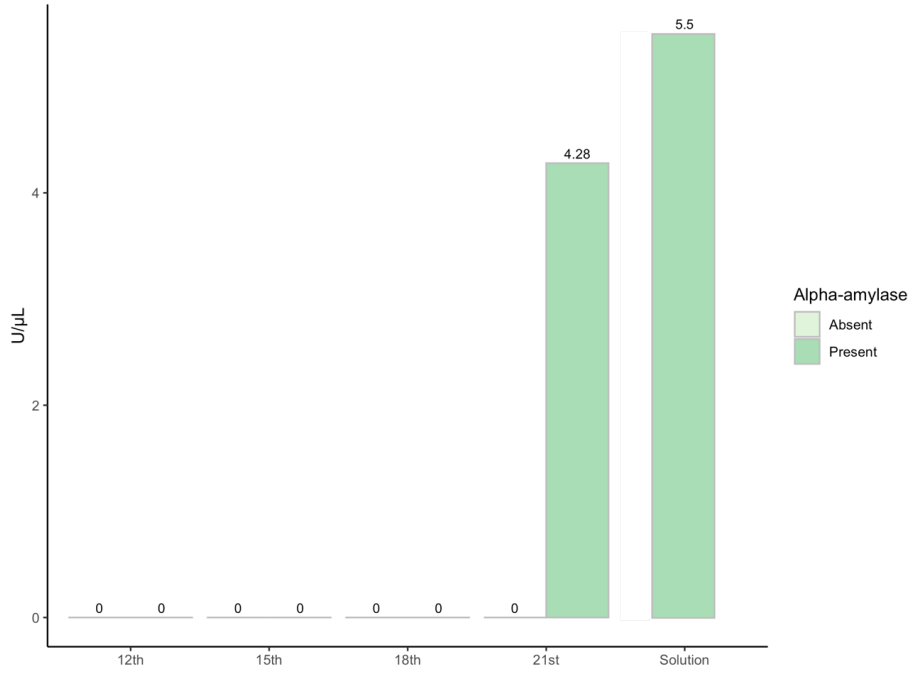

Figure 4. 1: The activity (U/µL enzyme) of alpha-amylase throughout the experiment from two of the calculus samples analysed and the artificial saliva solution with alpha-amylase. The values expressed represent the units of maltose released for every µL of the enzyme. The results obtained from the samples with and without alpha-amylase are here compared for days 12th, 15th, 18th and 21st, the values of alpha-amylase activity from the artificial saliva solution are here shown as a reference. This bar chart highlights the activity of the enzyme after two days of coexistence with the oral biofilm (on days 12th, 15th and 18th of the experiment) and after two days (on day 21st of the experiment).

In addition, as expected, no activity is detected in any of the specimens which were not inoculated with the enzyme (see Tables 1 and 2 in chapter 3). For completeness, it is important to note that no difference in amylase activity values is present between the samples containing the two starch granule types, as well as those native and boiled. Even if fluctuations are observable between these results, they are not sufficient to describe a fixed behaviour of the enzyme, and, therefore, these variables do not seem to constitute

discriminative factors in the persistence of the alpha-amylase when in contact with oral bacteria.

4.2 Effect of alpha-amylase and cooking process on sample weight

The dry weight of every dental calculus sample has been calculated and particular attention has been paid to the presence of alpha-amylase and processed starch granules in relation to the mass of the specimens. Namely, the mean of dental calculus weight has been determined by grouping the samples based on the starch solution used and the presence or absence of alpha-amylase. According to the result obtained (Table 4. 1 and Table 4. 2), a slight relationship between the weight of the samples and the presence of the degrading enzyme is visible (Figure 4. 2).

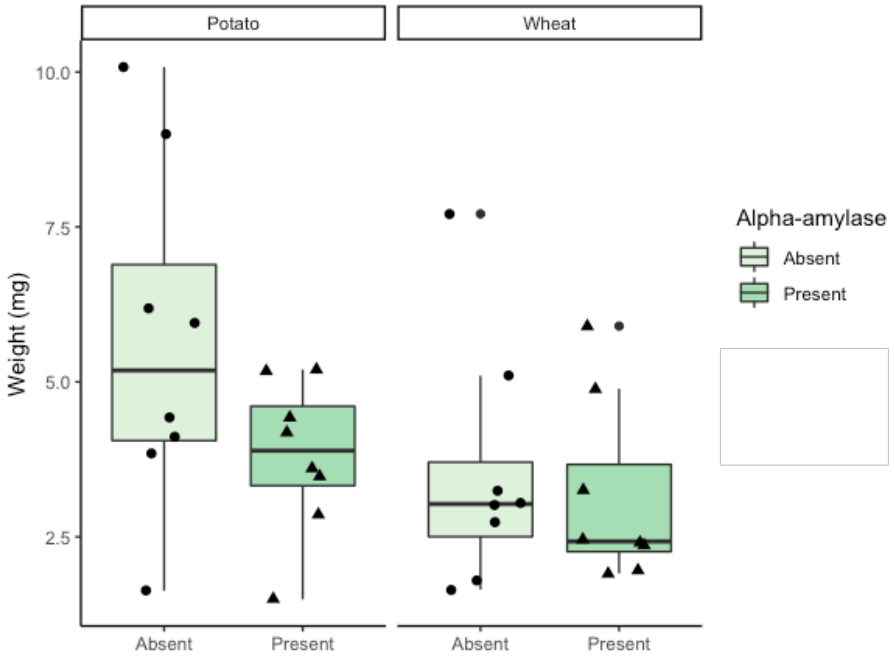

Figure 4. 2: Dry-weight in mg of the samples divided for starch granule type and presence of alpha-amylase.

In both potato and wheat treatments and regardless of the state of cooking of the starch granules, which results in the complete gelatinization of the granules, it appears that the dental calculus samples formed in the presence of alpha-amylase show a mean weight which is lower compared to dental calculus grown without the enzyme (Table 4. 1 and Table 4. 2). There is no relationship between the dry weight of the samples and the

processing of the starch granules (see appendix B); the cooking of the latter does not seem to affect the dental calculus mass in the analysed specimens.

Table 4. 1: Summary statistics for dental calculus weight (in mg) in samples with native starch treatment.

	Amylase	Mean	SD	Min	Max
Potato	Present	3.61	1.65	1.49	5.2
Potato	Absent	6.39	1.90	4.42	9
Wheat	Present	2.78	1.42	1.91	4.89
Wheat	Absent	3.26	1.41	1.65	5.1

Table 4. 2: Summary statistics for dental calculus weight (in mg) in samples with processed starch treatment.

	Amylase	Mean	SD	Min	Max
Potato	Present	3.38	1.01	2.86	5.17
Potato	Absent	4.92	3.61	1.63	10.08
Wheat	Present	3.5	1.64	2.4	5.9
Wheat	Absent	3.8	2.65	1.79	7.71

4.3 Starch granules counts

Since the counting process was carried out on three out of twenty-nine transects of the microscope slide, in order to extrapolate the total number of starch granules, and thus obtain the correct counts, two formulae have been employed, one for the specimens and one for the solutions. The first formula has the aim of calculating the total amount of starch granules present in the 100-200µL of EDTA in which the samples were dissolved; the starch granules embedded in the dental calculus samples are indeed now included in this solution. Since out of this volume only 20µL of material has been put on the slide and analyzed, the starch granules number of the entire sample needs to be extrapolated.

$$\text{Correct count in calculus} = \text{raw count x (portion of slide)}^{-1} \text{ x } \frac{\text{volume sample}}{\text{volume slide}}$$

In order to obtain the total number of starch granules to which the samples were exposed during the experiment, 10µL of each solution was mounted on a slide with 10µL of 20% (v/v) glycerol, and the number and size of the granules detected were recorded. The total

count was then inferred by multiplying the number obtained by 100µL to extrapolate the count to 1mL of solution and by the 16 days in which the treatment was carried out (Table 4. 3).

$$\text{Correct count in starch solution} = \text{raw count x } \frac{\text{total slides}}{\text{counted slides}} \text{ x 100 µL x 16 days}$$

The corrected counts obtained from the two formulae can be summarised as follow:

Table 4. 3: Corrected count of starch granules in the solutions grouped by different sizes: small (>10µm), medium (10-20µm) and large (<20µm)

	Small (%)	**Medium (%)**	**Large (%)**	**Total (%)**
Potato	4160000 (5.06%)	47680000 (58.08%)	30240000 (36.84%)	82080000 (100%)
Wheat	509600000 (75,79%)	158880000 (23,63%)	3840000 (0.57%)	672320000 (100%)

From the analysis of processed sample slides, it was possible to observe the complete gelatinization of the entire quantity of granules in the starch solutions, for both potato and wheat. As a result, these specimens were excluded from the following analyses since no starches were left to investigate. On the other hand, both potato and wheat native starch solutions show large counts of granules.

4.3.1 Potato starch

Eight dental calculus samples in total were analysed among those treated with potato starch solutions (*Figure 4. 1*). Four of them were exposed to alpha-amylase and the remaining four were not. For alpha-amylase samples, three show a similar starch count with a minimum of 0 and a maximum of 11 starch granules, while the fourth reveals a number of starches ranging from 23 to 97, and thus clearly higher than the previous three samples. Because of the particularly high count obtained from the last sample, this has been excluded from the analysis. The reason why such a high number of starch granules have been found in it is not completely understood and it could be related to the laboratory settings and the failure of oral bacteria in bonding with the enzyme.

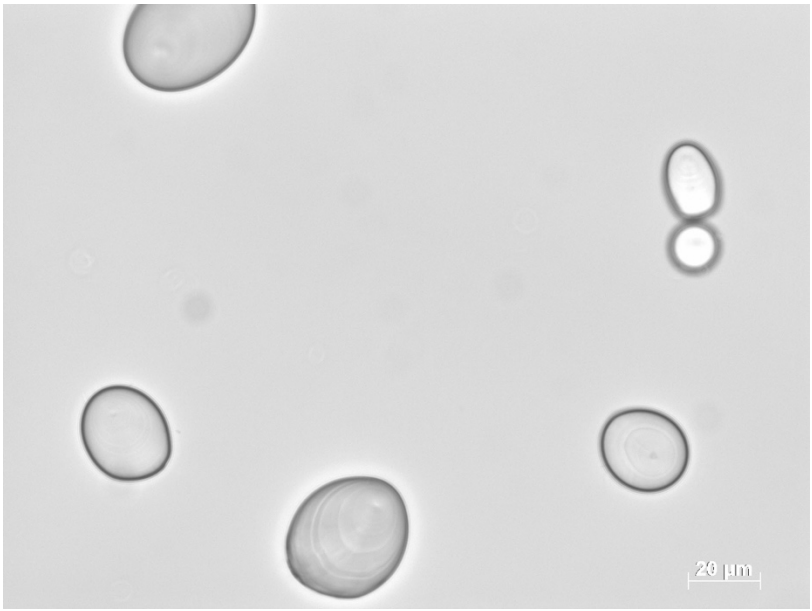

Figure 4. 3: Native potato starch granules of different sizes from the solution with unprocessed potato starches (picture taken by the author).

The native starch samples appear to be well preserved within the mineralized plaque, and do not show any trace of degradation. Dental calculus samples which formed within an amylase rich environment show a mean of 418 starch granules embedded within the matrix. The medium size appears to be the most abundant with 50% of the total number, on the other hand, with only 16 granules, the large granules are the less present. The samples that did not have contact with the enzyme show a number of starch granules comparatively higher than those previously described. Among all, the granules that seem to have been trapped with the highest frequency are those with a medium size (67.05%), while the least present starch group is represented by the large granules, showing a tendency similar to the samples with alpha-amylase (Figure 4.5).

4.3.2 Wheat starch

The total number of analysed samples that have undergone wheat treatment (Figure 4. 4) is eight. As with potato starch, four of them formed without having contact with alpha-amylase, while the other four grew in artificial saliva enriched with the enzyme. None of the observed slides of the amylase group retained a single starch granule.

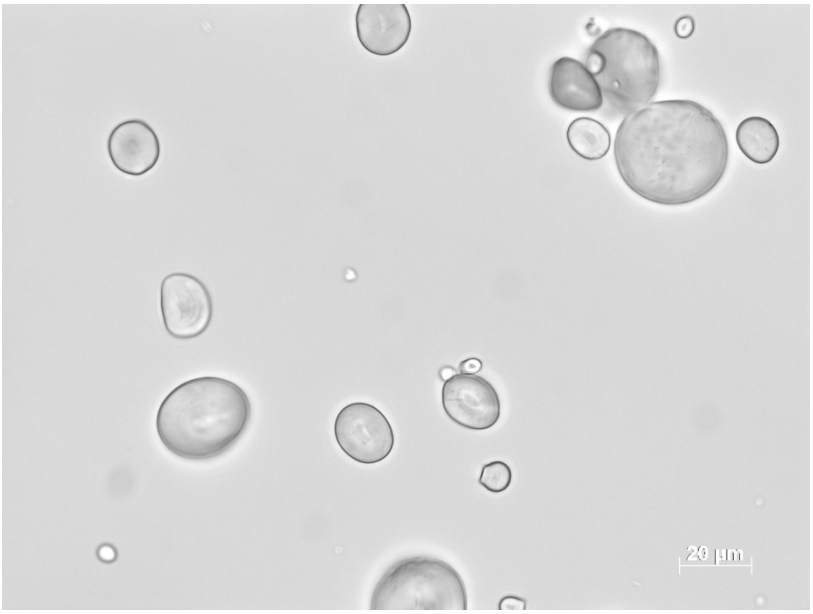

Figure 4. 4: Native wheat starch granules of different sizes from the solution with unprocessed wheat starches (picture taken by the author).

In contrast, the sample without alpha-amylase represents the category with the largest number of starch granules preserved with a total of 22860 elements. 96.83% of the whole amount is represented by the small size, which is also the most frequent in the solution count, while the less present group is composed of the large granules, with only 0.15% of the total number (Figure 4. 5).

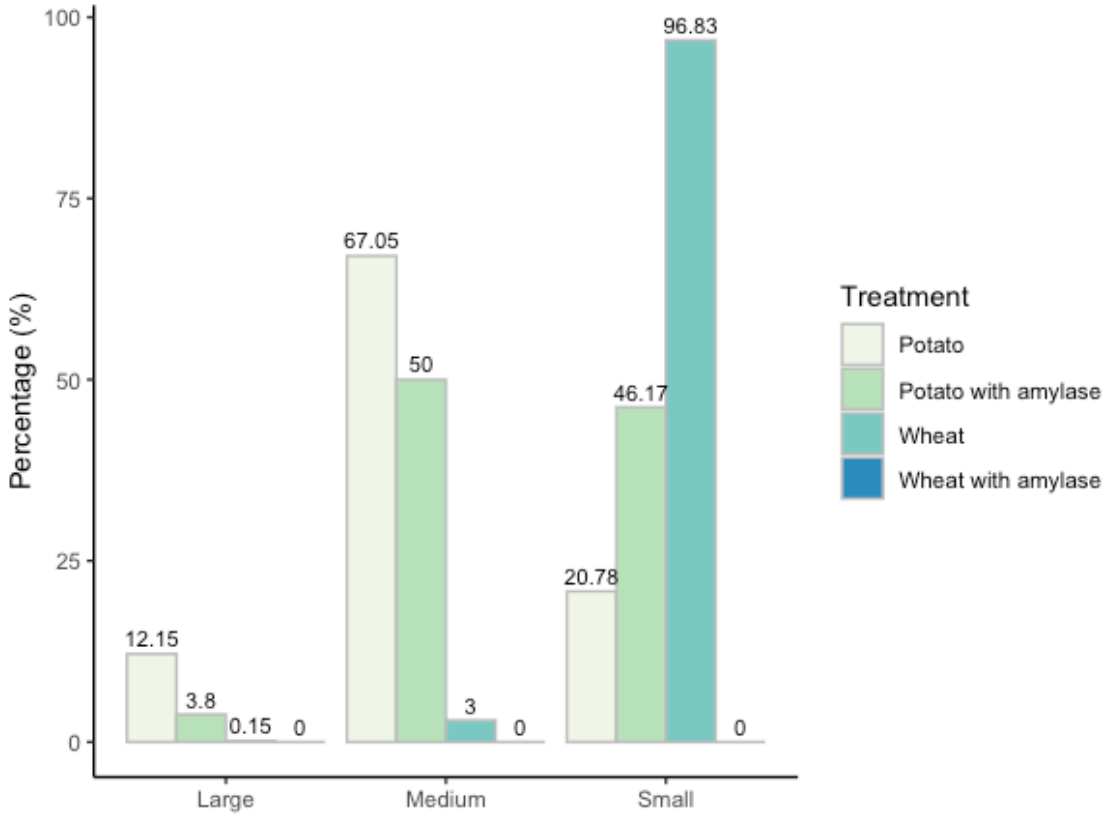

Figure 4. 5: Percentages of different starch granules sizes embedded in dental calculus grouped by the type of treatment. The values used for this bar chart are reported in Appendix C.

4.4 Starch percentage in dental calculus

Once the total starch counts were extrapolated from both the samples and the solutions, the mean percentage of granules incorporated into the dental calculus matrix over the course of the treatment was estimated based on the total number of starch granules to which the samples have been exposed (Table 4. 4).

Table 4. 4: Mean percentage of starch granules trapped in dental calculus matrix from the solutions, grouped by different size: small (>10µm), medium (10-20µm) and large (<20µm).

	Amylase	Small (%)	Medium (%)	Large (%)	Total (%)
Potato	Present	0.004%	0.000%	0.000%	0.004%
Potato	Absent	0.011%	0.003%	0.000%	0.014%
Wheat	Present	0%	0%	0%	0%

The results obtained from this calculation show a clear difference in the percentages of starch granules based on the presence of alpha-amylase. As regards potato starch, the absence of the enzyme during the process of dental calculus formation enables 0.010% more granules to be trapped within the dental calculus matrix. For both the potato solutions, the granules that tend to be more easily included in the mineralised plaque are smaller than 10µm, a behaviour which is shown to be the same for wheat starch granules. When alpha-amylase is present, none of the wheat molecules survived in order to be recovered in dental calculus, while in potato samples only a tiny portion of the small granules was preserved. Instead, when there is no contact between the granules and the enzyme, the degradation process does not occur, and the food particles were more prone to dental calculus embedding. Almost the total amount of starch granules that got trapped within the matrix showed a size below 10µm. This represents 0.004% of the total starch granules exposed to the oral biofilm throughout the experiment (Table 4.5). The prevalence of granules below 10µm embedded in the dental calculus is a repeated pattern that characterises both potato and wheat starch granules.

4.5 Chapter summary

This section of the study has been dedicated to the description of the results provided by the microscopic observation of the samples and the laboratory analyses carried out in regard to the alpha-amylase activity. The outcomes described show some clear patterns of the starch granules behaviour in relation to the variables to which they are exposed. Starting from the dry weight of the mineralized biofilm, the results show relatively higher values in the samples which were not exposed to alpha-amylase, while no correlation is found between the mass of dental calculus and the type of starch exposed to it. Moreover, the detection of the enzyme activity highlighted a clear degradation after

only three days of interaction between the oral bacteria and the alpha-amylase itself, behaviour which will be better investigated in the next chapter.

Finally, the focus has moved to the embedding of starch granules within the dental calculus matrix, providing patterns of inclusion in relation to the variables to which the samples were exposed. Namely, for potato starch, middle size granules appear to be the most present in the mineralized dental plaque, with a lower amount of food fragments in the specimens exposed to alpha-amylase. Moreover, wheat starch granules are almost only recovered in small size and show a complete degradation when subjected to alpha-amylase. Finally, the percentages of the starch granules incorporated have been calculated for every variable, thus allowing a clear understanding of the process. The interpretation of the results here obtained will be discussed in the next chapter.

5. Discussion

As already mentioned, even if not harmful *per se* (Tan et al., 2004), dental calculus is considered to be part of the broader group of diseases studied in paleopathology (Waldron, 2008). For several years, this mineralized plaque has been seen only as a secondary cause for periodontal disease (Albandar et al., 1996), and frequently removed from dentition because obscuring traits regarded as more important from an archaeological perception. Nowadays, dental calculus has gained popularity thanks to its capability of preserving important information from the past within its matrix (MacKenzie et al., 2021), and its analysis is widely used for archaeological investigation of the human past. Particular attention has been paid to the dietary insights that this material is able to provide and within the last fifteen years a lot of publications have been released with the aim of bringing to light direct evidence of the human past which were not accessible until then (e.g., Gismondi et al., 2018; Mann et al., 2020; Wesolowski et al., 2010)

However, the process that leads to the formation of dental calculus is not fully clear and it may represent an obstacle to the interpretation of the archaeological evidence (Lieverse, 1999). Namely, as reported in many previous studies with both non-human primates and living human populations (e.g., Leonard et al., 2015; Power et al., 2015), starch granules, which are commonly found in dental calculus, are considered to not follow a defined pattern in the process of incorporation into calculus matrix, which leads to over and under-representation of these dietary indicators. The mechanism by which these food particles are incorporated into the mineralised dental plaque is largely unknown and very few studies provide a tentative explanation. The problem which arises from this background is related to a wrong or biased interpretation of archaeological evidence (Leonard et al., 2015), and the results obtained from it can be only linked to the type of food eaten, but not to the quantity or the quality. The experiment carried out for this research provides a reliable source of information regarding the process of starch embedding, thus indirectly enabling a better understanding of the archaeological samples.

In this framework, the goal of this thesis and, particularly, the purpose of this chapter is to explore the results obtained from the summary statistics of both the starch count and the alpha-amylase activity detection. These two aspects of the study will then be put into context in order to obtain a bigger picture of the dental calculus analysis. From the data collected, at first sight, it is possible to infer conclusions on the process that let starch granules be embedded into the calculus matrix, but it is only by considering every possible variable that the results can be properly interpreted. To be able to reach such conclusions, every aspect of the experiment will be analysed and eventually put together to create an overall view of the starch granules' behaviour related to dental calculus embedding. Finally, comparisons with the study of Bartholdy and Henry (2021) will be carried out to evaluate the stochasticity of the scenario described.

As stated in Section 1.3, this research had the aim of assessing the potential of starch granules to provide information on dietary habits of past human populations when extracted from dental calculus. In order to provide an answer to this issue, in the first section of this chapter, the differences identified in the embedding behaviour of potato and wheat granules will be investigated with a focus on their size and the possible reasons that led to the results obtained from the dental calculus starch counts. In addition, the role of alpha-amylase in the process will be analysed with the aim of providing a clear evaluation of its role in the starch degradation process that occurs in the oral environment (Scannapieco et al., 1993). The enzyme and how it affected starch throughout the experiment will be eventually described on the basis of the results obtained from the amylase activity assays, and its persistence in the experiment will be further evaluated.

Finally, when the research questions of this study are fully addressed, this chapter will present all the limitations that were identified while conducting the experiment, and the constraints that the interpretation itself presents when approaching this research in the archaeological context. The focus will be based on the variables which occur in normal conditions and are thus present in archaeological samples, which nevertheless cannot be reproduced in the experimental setting. In fact, mechanical actions carried out by the individuals such as oral hygiene and particles removal by the tongue (Kashket et al., 1996), are very hard to recreate in a simplified *in vitro* model.

5.1 The behaviour of starch granules in dental calculus

The results obtained from the counts of the starch granules in dental calculus show a different behaviour based on the variables added to the samples, indicating a possible relationship between the starches themselves and the different aspects to which they were exposed. In particular, the species and the dimension of starch granules employed for the experiment appear to be great discriminative factors on the final counts.

In order to assess the consistency of the present study, a comparison with the research of Bartholdy and Henry[1] (2021) will be made focusing on the samples which were not exposed to alpha-amylase in the current research. The two experiments, indeed, were mostly carried out by following the same protocols, and thus the results are expected to be consistent with each other. In both investigations, potato starches appear to have greater success in the inclusion within the mineralized dental plaque, showing a higher representation of the small food microfossils among the other sizes. On the contrary, the outcomes from this study on wheat granules seem to disagree with those of Bartholdy and Henry in which the medium debris are more abundant than the others. Nonetheless, the researchers match on defining a general greater embedding success in potato starch granules rather than in wheat and underrepresentation of the molecules larger than 20 μm in both studies. Finally, it is important to point out that the percentages of the two granule types recovered within the calculus in the present investigation, appear to be generally lower compared to those obtained in the previous analysis. This small divergence experienced in the percentage of starch granules embedded in the entire sample population can be attributed to intra-variation differences that occurred during the execution of the protocol, in fact, these factors could have modified the response of the starch granules themselves. In addition, the difference in the outcomes obtained from the two experiments can be caused by the microbiota; in fact, in the present study, the whole saliva was collected from two donors, the author and Bartholdy, while in Bartholdy and Henry's research only Bartholdy contributed. Thus, a divergence in bacteria type and number in the oral biofilms could have led to different results in the quantity of starch granules recovered in the final mineralized samples.

[1] Bartholdy and Henry (2021), by following the protocol developed by Sissons and colleagues (1991), analysed the embedding process of wheat and starch native granules within *in vitro* mineralized plaque. The study focused on three different starch solutions, one containing potato granules, one containing wheat granules and the last one containing a mix of both wheat and potato granules. The whole experiment was carried out without adding alpha-amylase.

Although not with complete confidence, the similarities provided by the two studies can somehow prove a certain degree of repetitiveness in the process of embedding starch granules within the dental calculus matrix. As we can assess from the results, in native molecules, the tendency of incorporation is highly demonstrated for those showing a small to medium size, compared to the granules with a dimension larger than 20µm. This result sheds light on the underrepresentation of larger starch granules and suggests a higher presence of small food microfossils in the archaeological mineralized plaque. Consequently, starch granules which naturally occur in greater size can be less represented in both human and animal remains recovered from archaeological contexts, without however necessarily being related to reduced consumption of them by the past populations. In addition, it is important to note that the content of amylose and amylopectin in starch granules is considered to change accordingly to the size of the granule itself. Larger granules usually show larger amylose content which can lead to a higher level of degradation compared to smaller starches (Fuentes et al., 2019).

5.1.1 The importance of starch granule taxa

As previously mentioned, every starch granule presents different biological characteristics that are highly dependent on the plants in which they are produced. In particular, the starches selected for this study, potato and wheat, show really clear variations between each other; they are respectively a tuber and cereal grass. When observed under a microscope (Gismondi et al., 2019; Jane et al., 1994; Van Velde et al., 2002), they present substantial differences from each other in size, shape and surface area; potato granules are ovoid to pyriform in shape and can reach up to 100µm in length, with a maximum width of 70µm, in addition, they show a larger volume compared to their surface area. On the other hand, wheat starches present a large lenticular and smaller spherical shapes, a size of a maximum of 35µm and a surface bigger in relation to their total volume.

The potato and wheat starch granules show substantial differences in their behaviour when exposed to the same variables in the oral biofilm model. Since the experimental conditions applied are the same for both the starches, the reason for the dissimilar inclusion patterns can be attributable to the physical differences between the two types of granules. The amylose-amylopectin ratio which is characteristic of every starch type is deemed to impact the physical properties of the granules (Torrence & Barton, 2006), thus

creating a difference in their response to the oral environment. This ratio is based on both the genetics of the plant and the environment in which it grows, making this difference even more pronounced when applied to the natural setting.

It is important to consider that the physical properties which differentiate potato and wheat granules are much more diverse in natural conditions. Several factors affect the production and growth of starches within the plants and based on the region in which the plant grows, these can be meaningful to various extent. Time, temperature, stress and nutrition can create differentiation in starch granules production between plants (Torrence & Barton, 2006) thus causing even more divergence when addressing the preservation and embedding in dental calculus matrix.

5.1.2 The importance of starch granule size

During the course of the analysis, a lot of attention has been paid to the different sizes of the starch granules. It has been demonstrated that the various dimensions of starch granules are highly dependent on the CO_2 concentration present in the growing environment and thus they are extremely related to the plant species (García-Granero, 2020). As previously described, the counting process was based on three categories related to the dimension of the molecules, and the same classification was also kept for the interpretation of the results.

From the count number provided here, it is clear how in every sample analysed the largest granules are underrepresented compared to the total amount recovered in the original solutions, demonstrating a much higher embedding of fragments below 20 μm. Smaller granules, on the contrary, are the highest represented granules trapped in the samples and, as shown in the results, a bigger percentage of them made it into the dental calculus matrix compared to the larger molecules (Figure 5.1). This repeated pattern of incorporation can be justified by suggesting greater ease in the action of embedding by smaller granules due to their physical characteristics.

The size of the starch granules has been previously deemed to be an important factor during the process of hydrolysis carried out by alpha-amylase. Namely, the smaller the dimension of the food debris, the higher is the effect that the enzymatic digestion has on it (Qi & Tester, 2016). This occurs due to the fact that when the surface area of the granule itself is larger compared to the volume ratio, a wider area is available to alpha-amylase

for implementing hydrolysis (Tester et al., 2006). The stronger effect that the enzyme produces on the smaller starch granules compared to those with bigger dimensions is also provided by the counts obtained in the present study with regard to wheat samples. When addressing the difference in the starch number for the oral biofilm exposed to wheat granules, the large number of small molecules makes them the most affected by the alpha-amylase hydrolysis, confirming the previous research (e.g., Franco et al., 1992; Haslam, 2004).

However, the same behaviour is not observable when dealing with potato starch granules; by analysing the percentages of granules exposed to the degrading enzyme, those with a size bigger than 20 μm appear to be the most preserved category. This outcome can be justified by the structure of the potato starch granules; this is constituted by an elaborated combination of hard and soft materials which make the molecules more resistant to alpha-amylase hydrolysis than other starch species (Gallant et al., 1992).

5.2 Alpha-amylase in *in vitro* dental calculus

Salivary alpha-amylase is considered to be the most abundant constituent of human saliva, and an essential enzyme for the digestion of food (Zhang et al., 2022). In the oral environment, this enzyme plays two fundamental biological roles: the first, as previously described, is to break down the chains by which the carbohydrates are constituted, in order to obtain smaller and easily digestible molecules. Namely, these new products, in the case of alpha-amylase, are glucose, maltose and maltotriose (MacGregor, 1988). The second function carried out by the enzyme is strictly related to the bacteria present in the mouth, in particular, streptococci. Oral streptococci, also called alpha-amylase binding streptococci, are known to be one of the first species to settle on the biofilm which lays on the tooth surface, and they seem to stimulate its colonization by other types of bacteria (Nikitkova et al., 2013, p. 416), thus increasing the growth of microorganisms population in the dental plaque. As the name suggests, these bacteria bind with the enzyme, allowing them to better survive in, and contribute to the oral environment.

In humans, alpha-amylase is constantly produced by salivary glands under the regulation of the nervous system (Rohleder & Nater, 2009, p. 470), and because of this, it is highly sensitive to external factors such as stress and exercise. On the contrary, the enzyme used for this study was added only once to the artificial saliva after having verified its

preservation over time. Therefore, every change suffered by alpha-amylase throughout the experiment can only be a direct cause of the interaction with the recreated oral environment (i.e., bacteria and starch granules). As shown in the results, the activity of the enzyme seems to decrease significantly on the third day of coexistence with the oral biofilm, providing a clear repetitive behaviour. Two days after the medium replacement, however, starch granules appear to be subject to a level of enzymatic activity which matches the original values, providing the existence of a clear critical point in which the alpha-amylase activity is strongly reduced. The results obtained in the granules counts show a significant difference when the variable of alpha-amylase is added to the samples, demonstrating some level of starch digestion.

Although the current study cannot find a justification for this enzymatic behaviour, the introduction of fresh artificial saliva every three days enabled the preservation of the interface between the starch granules and the alpha-amylase, making it possible to resume the digestive process.

5.2.1 Alpha-amylase as biofilm inhibitor

Thanks to the comparison between the dental calculus samples, it was possible to observe a consistent lower dry-weight of those exposed to alpha-amylase compared to the mineralised dental plaque which did not come into contact with the enzyme (see Figure 4.2). This result can be associated with the activity of alpha-amylase to act as an inhibitor for the formation of biofilm bacteria which help the formation of the biofilm itself (Lahiri et al., 2021). *In vitro* studies have demonstrated that the presence of the enzyme causes the decrease of specific bacteria and thus a less amount of oral biofilm. The outcomes obtained from the comparison of the samples dry-weight for both potato and wheat starch granules which sees general lower values in dental calculus exposed to alpha-amylase can be considered as the result of the enzyme activity which acted as an inhibitor for the formation of the biofilm.

5.3 Processed starch granules

As explained in the results chapter, the solutions with the processed potato and wheat starch granules did not preserve any of the incorporated particles. The water combined with the heating and stirring activities proved fatal for the integrity of both the

granule types, resulting in the complete gelatinization that prevents their identification in the solutions and consequently, in the mineralized biofilm. It has been previously demonstrated by several studies that different cooking processes lead to irreversible damage of the starch granules (Barton & Torrence, 2015; Henry et al., 2009; Wang et al., 2013), but the results of the cooking process can be various and are strictly connected to the raw material (Crowther, 2012).

During the last years, a wide number of cooking methods have been tested on starchy plants in order to assess how the granules behave in the different processing activities. This research has been carried out with the aim of providing clear identification of both the starch granules and the cooking process in archaeological remains, thus obtaining insight into the habits of human populations in relation to diet. The results obtained in the literature by boiling different food containing starch granules are consistent and show the collapse of the granules with severe swelling and the change or loss of the extinction cross typically observable under the polarized light (Eliasson, 1980). However, this reaction seems to occur only after a long cooking time, ca.60 minutes (Henry et al., 2009), and only if the boiling process takes place in more than 60-65% (*w/w*) of water (Wang et al., 1991) at a temperature around 60 – 85 °C. Before reaching this state, the starch granules undergo a degradation process (*Figure 5. 1*) in which they slowly get swelled, lose the birefringence, and eventually completely gelatinize based on the period to which they are cooked (Crowther, 2012; Henry et al., 2009); the longer the food is cooked, higher are the probabilities that the starch granules got damaged.

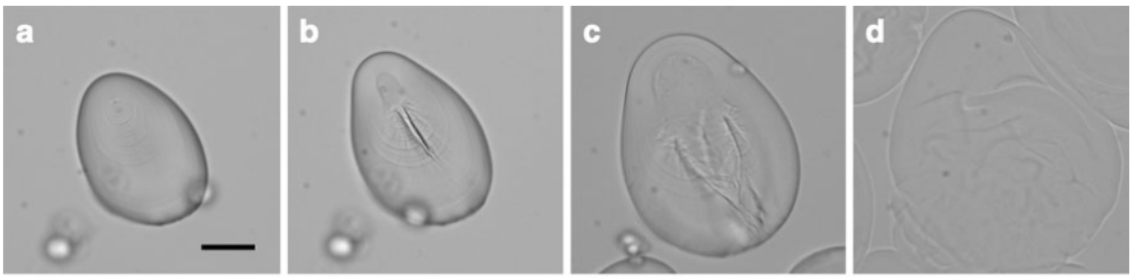

Figure 5. 1: The process of gelatinization of a starch granule of potato (Solanum tuberosum) from Crowther (2012, p. 223). Native starch granule (a); partially gelatinized granule in two different phases (b, c); completely gelatinized granule (d).

Moreover, other factors to keep in mind when analysing processed starch granules from an archaeological context are the type of plant from which they originate and the modality

in which the food is presented (e.g., flours, grains, tubers). In fact, it has been widely demonstrated that every starch granule acts differently based on its taxon, and thus physical properties (García-Granero, 2020), and that the conformation of the food itself can preserve or improve the exposition of granules to the gelatinization process (Crowther, 2012). Namely, it frequently happens that when mixed with other substances for food preparation, starch extracts tend to create aggregates (Herrera-Gómez et al., 2002) in which only the outer area is blended with water and thus the inner part is protected from degradation. The gelatinized starch granules on the outside protect the native ones by creating an impediment and thus preventing them to undergo modification. On the other hand, gelatinisation in whole foods is mostly based on the moisture that the plant itself presents, which is higher for tubers and roots, and lower for grains (Peroni et al., 2006). The natural quantity of moisture contained in tubers and roots is considered to be enough for the gelatinisation of their starch granules during the cooking process, while in grains the degradation is highly dependent on the water and heat used (Stapley et al., 1998). The result of this different behaviour on the basis of the physical structure of the starchy plants adds a new factor to the interpretation of the archaeological evidence.

According to what has just been said, the retrieval of native starch granules in dental calculus cannot confidently be interpreted as the consumption of raw food; as described, these food particles can be protected from the gelatinization process while being cooked based on how the plants occur in nature or how they are modified by human beings. Consequently, when approaching dental calculus analysis it is important to take into consideration the natural behaviour of starch granules and how they could react to cooking processes in order to avoid biased interpretation of the archaeological data.

5.3.1 The behaviour in the laboratory setting

When comparing the outcomes of previous analyses with the gelatinized starch granules resulting from the processed solutions in this study, it is important to consider that the described literature only addresses the investigation of entire plants, grains, flours and tubers, and not directly industrially separated starch preparations as were used in the present research. When in whole seeds, wheat starches were still visible, if damaged, after 40 minutes of boiling (Henry et al, 2009). Isolated and damaged starch grains, such as in commercially-produced starch powders, are more fragile compared to the native ones, and thus more liable to both mechanical agitation and microbial attack (Barton, 2007).

Due to water and heat, the granules swelled and reached a state of high vulnerability, which together with the continuous stirring process could have caused them to burst and break up into smaller pieces.

The gelatinization of the food particles in the two boiled solutions, if on the one hand represents a failure for the conducting experiment, preventing the analysis of processed starch embedding in dental calculus, on the other hand, it allows gaining insights into the behaviour of cooked granules when these are not preserved by the plant structure itself. From this outcome, we can thus assess that starch extracted from plants, which is usually used in research could not act as it would normally do in a conventional environment, and therefore particular attention should be paid when this material is used in laboratories for replicating natural phenomena.

5.4 The contribution to archaeological research

As stated in Chapter 1, one of the main purposes of this study is to overcome the biases in the analysis of archaeological dental calculus is one of the main purposes of this study. The poor knowledge of the mineralized plaque as a pitfall for plant microfossils makes the interpretation of the dietary evidence challenging. This experimental investigation had the aim of shedding light into the process of starch granules embedding within the calculus matrix, in order to allow a clearer reading into the human past habits. Although we are not fully able to provide a good explanation of the mechanism of starch inclusion, a few good examples have been provided for a better interpretation of the archaeological data.

This investigation has indeed demonstrated the presence of a pattern in the incorporation of starch granules, thus contradicting the belief of some kind of stochasticity in this process (Leonard et al., 2015; Power et al., 2015). Indeed, even if it is correct to support a general underrepresentation of the starch evidence found in the calculus compared to the actual intake (Kashket et al., 1996), this study, together with the research carried out by Bartholdy and Henry (2021), shows how the potato and wheat granules follow a specific behaviour during the embedding into the calculus matrix. The high level of control over the experimental conditions enabled us to avoid the typical variables which occur *in vivo* and are often the case for misleading results. These factors, which include

the inter- and intra-individual variations, strongly influence the analysis and can make the interpretation difficult from an archaeological point of view (Leonard et al., 2015).

Taking into consideration the limitations to which this method is prone, which will be further discussed, it represents a convenient protocol for a better understanding of the mechanisms which take action in the oral environment. The development of such a process and the control of the different variables can provide practical training for a better understanding of the dental calculus analysis, without the need of employ archaeological remains or living populations.

5.5 Limitations

In osteological research, it is custom to assess the aspects and the factors which prevent a clear interpretation of the archaeological assemblage. Therefore, with the aim of taking consciousness of the constraints that this study presents, in the next sections a wide assessment and description of its limitations will be provided. In order to be as explanatory as possible, attention will be given to both the experiment carried out for this study with its implication for archaeological interpretation and the analysis of dental calculus conducted on osteological samples. With the aim of providing a bigger picture of the main topic, a first description of the limitations of osteoarchaeology as a discipline will be outlined.

5.5.1 Limitations of dietary research with archaeological dental calculus

It has been mentioned in the introduction of this thesis that the analysis of dental calculus from archaeological contexts has lately gained popularity due to its potential in the preservation of food microfossils. The position of witness of the past played by the mineralized dental plaque has increasingly earned it the role of mine of information on the human past, from diet-related insights, thanks to the plant microfossils embedded in dental calculus, to genetic knowledge which derives from the microorganisms recovered within the material (Radini & Nikita, 2022).

In regard to the use of dental calculus for the retrieval of food consumption data, various limitations can be encountered. As already mentioned in this thesis, the process that leads to the embedding of food particles within the calculus matrix is not yet fully understood,

and it decreases the degree of confidence in the analysis of this archaeological evidence (Fox et al., 1996). Throughout the last fifteen years, the critical aspects of mineralized dental plaque as a source for the investigation of dietary habits have emerged from the research. Leonard and colleagues (2015) pointed out the bias which arises when comparing individuals of different ages; older people are statistically considered to show a higher amount of calculus deposit compared to the younger population. As a consequence, based on the excavated individuals the availability of the material can be restricted and somehow might not represent the entirety of the population under analysis. Moreover, as explained in the first chapter, there are several pathways by which food debris becomes included in dental calculus and they do not necessarily need to be the consequence of conscious ingestion of food (Radini et al., 2017)

It is important to note that the vast majority of dietary fragments are swallowed during food consumption, and thus only a small portion of them is available in the mouth to be embedded within the oral biofilm (Bartholdy & Henry, 2021). Finally, the retention of food particles is highly dependent on the viscosity of the consumed substance, and therefore, it is important to take into consideration that the larger presence of a specific typology of debris is not necessarily connected to the quantity assumed (Kashket et al., 1991).

5.5.2 Limitations of the experiment

The aim of this study was to replicate the normal condition of the dental calculus formation in human beings in order to investigate the process which leads different starch granules in the inclusion within the matrix of the mineralized plaque. Although the experimental setting in which this research took place allows a high degree of control, some methodological aspects did not enable to fully reproduce the *in vivo* scenario.

The use of artificial saliva with alpha-amylase only started on day 10^{th}, while the inoculation with whole saliva was carried out for the last time on day 5^{th}. This, therefore, prevented the exposure of the enzyme to the oral bacteria. The lack of direct interaction between the two can have led to a less degree of survival of the microorganisms; indeed, the bonding between alpha-amylase and a specific type of bacteria has proved to be necessary for their existence and adherence to the tooth surface (Nikitkova et al., 2013; Scannapieco et al., 1993). This could have thus resulted in different behaviour of both the

enzyme and the bacteria compared to how these two normally act in the oral cavity of an individual.

The use of EDTA for the dental calculus dissolution could have caused some changes between the size categories. Despite previous studies on this chemical pre-treatment have assessed its minor influence on the morphological modification of the starch granules (e.g., Le Moyne & Crowther, 2021; Modi et al., 2020; Tromp et al., 2017), these did not focus on the possible alteration suffered by the dimension ratio (Bartholdy & Henry, 2021). Therefore, it is plausible to consider the use of EDTA as a potential bias in the interpretation of the starch count. Nevertheless, it is advisable to further investigate the influence of chemical pre-treatment on the starch size in order to obtain a clearer picture of the situation.

Furthermore, one of the aspects which strongly limited the realistic nature of the human remains was the lack of the effect of the taphonomic processes which commonly occur in the archaeological contexts (Blau, 2017). Both dental calculus and starch granules have been proved to be highly liable to chemical and physical degradation due to external factors such as the burial conditions (e.g. Hutschenreuther et al., 2017). It is thereby inevitable to come to the conclusion which sees the absence of this variable in the experiment as a critical aspect that could have led to an overrepresentation of the starch granules, and therefore to a biased interpretation.

Finally, a limitation caused by the experimental nature of this study is the use of only two types of starch granules, potato and wheat. In order to be applicable to the archaeological remains and allow a good interpretation of the data, this study needs to be carried out on several more starch taxa such as legumes and wild plants. The variability of the human diet in an *in vivo* context is constituted by a wider range of starchy plants, and the present study only enables us to understand the behaviour of potato and wheat granules when engaging with the oral environment. In addition, the small sample size which characterizes this experiment represents a limitation for the interpretation of the results. The analysis of four samples for each variable does not provide a strong basis for the understanding of the starch granules incorporation in dental calculus and all the interpretations must be considered tentative.

5.6 Chapter summary

The interpretation of the results obtained from the starch counts outlined a repetitive pattern in the process of inclusion of the granules within the calculus matrix. The microfossils which show a size smaller than 20 μm are generally overrepresented in the samples compared to the larger granules of the same type, when not exposed to alpha-amylase. Once the enzymatic degradation takes place, the factor with the most influence appears to be plant source of the starch; indeed, potato and wheat granules display very different behaviour, probably due to their individual physical characteristics. In addition, both alpha-amylase and the cooking of native starch granules prove to engage particular behaviour when applied to an *in vitro* setting, diverging from the natural process.

Thanks to these results, it was possible to obtain new insights into the mechanism which drives the inclusion of particles within the mineralised dental plaque. The nature of the food molecules and the way in which they react to the oral environment has proved to be the key to a better understanding of the archaeological dental calculus. In addition, by comparing the samples exposed to alpha-amylase to those which did not suffer the enzymatic digestion, it was highlighted the essential role of degradation carried out by alpha-amylase itself, giving even greater emphasis to the peculiar behaviour of the starch, based on their species. Finally, even though the cooking of the native granules did not allow to get any valuable results from the dental calculus, interesting deductions were made which can help future analysis.

6. Conclusion

The main purpose of this study was to understand to which extent the food microfossils embedded in archaeological dental calculus are good evidence of food consumption among past human populations, with a specific focus on starch granules. The lack of clear knowledge in the process which leads to the inclusion of this kind of dietary fragments in the mineralized dental plaque is indeed a strong obstacle to a definite interpretation of the archaeological material. This study wanted to put itself as an attempt to better understand the behaviour of starchy remains in an oral biofilm model in order to thereby be able to apply these mechanisms to the interpretation of the archaeological context.

Even though we are not able to fully prove a standard mechanism in the embedding of starch particles in the *in vitro* dental calculus, the outcome raised from the analysis allowed us to outline some key factors taking place in the process. One of the major results of this study was the establishment of a clear repetitive pattern in the starch granules inclusion behaviour; this indeed proves the erroneousness of the idea by which starch fragments are embedded in the dental calculus matrix with a higher degree of stochasticity. Nonetheless, it is important to note that, even if a standard process was recognized, this was highly related to the type of granules and therefore, not applicable to the wholeness of the samples which took part in the investigation. In addition, the analysis of the controlled variables added to the study, such as the alpha-amylase and the granule types, enabled us to infer information from the different aspects which are involved in the process.

In this last section of the thesis, the last consideration of the results obtained will be made in order to finally answer the research questions outlined in Chapter 1. Moreover, a reassessment of the limitations of this study will be carried out with the purpose of describing the direction in which future studies may be targeted.

6.1 Addressing the research questions

The present study aimed to a clarification of the process of starch granules incorporation within dental calculus in order to be able to assess how much these particles are good proxies for the interpretation of dietary habits of human past populations. In order to finally assess this purpose, the behaviour of potato and wheat starch granules in an *in vitro* oral biofilm and their reaction to the different variables were analysed. The use of the biofilm model allowed us a great degree of control over the factors which normally affect the dental calculus formation and the embedding of food particles; as a consequence, this process determined the opportunity to avoid the inter- and intra-individual differences which commonly occur in a normal environment. In fact, it is this high level of variation which makes the analysis and the interpretation of dental calculus difficult to carry out, in both modern and archaeological contexts. Conducting the investigation in a laboratory setting thus allowed us not only to have good control over the oral biofilm formation and mineralization but also to test how the single variables influenced the outcome of the experiment.

The starch granules retrieved from the dental calculus at the end of the experiment, in general, show a great level of dependence on the basis of the variables added to the samples. Namely, the starch molecules behaved differently in accordance with their species when they were exposed to alpha-amylase. While a general underrepresentation of bigger starch granules (> 20μm) is recognized for both potato and wheat starches in the calculus matrix when the enzyme is not present, the same result did not occur when alpha-amylase is present during the oral biofilm growth. It is precisely when the digestive enzyme takes action that the behaviour of the starch molecules differs on the basis of the species to which they belong. This outcome can be traced back to the peculiar individual properties which potato and wheat granules present, such as size, surface area and crystalline structure.

The influence of alpha-amylase in the inclusion of starch molecules within the dental calculus showed to be an important discriminating factor. Both potato and wheat granules were deeply affected by the enzymatic process, showing, however, a somewhat different reaction. While potato microfossils count in dental calculus was smaller compared to the samples which were not exposed to alpha-amylase, with a higher impact on the bigger granules, the wheat molecules were completely hydrolysed by the enzyme, thus preventing their embedding within the matrix of the oral biofilm. The reaction

implemented by the two starch types sheds light on the great importance of the alpha-amylase digestive process which takes place in the oral environment and in the impact that it has on the granules incorporation. Furthermore, as previously stated, the clear influence represented by the species of the starch granules is here clearly exposed and highlights the importance of interpreting the food remains in dental calculus on the basis of the plant to which they belong.

Finally, because of the gelatinization of the starch granules during the cooking process, which prevented their count in the mineralized dental plaque, it was not possible to assess how the damaged structure of the molecules can influence their inclusion in the calculus matrix. Nevertheless, thanks to this unfortunate event, it was possible to determine how the behaviour of the starch extract normally used in the laboratory differs from the conduct of starch granules embedded in food. It was indeed possible to determine that the food structure acts as a barrier from heat and water, protecting to a certain level the starch present in it. On the contrary, this process does not occur in the starch used in the laboratories, and this results in starch granules which are more sensitive and prone to a faster modification and degradation.

6.2 Direction for future studies

The limitations which affect both this experiment and, in general, the dietary analysis of archaeological dental calculus are widely explained in Section 5.5. With regard to this study, the encountered constraints were almost only related to methodological aspects; albeit the present research has set itself bold purposes, it represents one of the few on this specific topic, therefore suffering from the lack of well-defined methodology. In order to assess and increase the validity of this experiment, it would be desirable to proceed with its implementation.

One of the most important aspects which affected this study was the gelatinization of the starch granules which occurred during their cooking; despite the adoption of measures in order to avoid the degradation of the starch molecules, such as the cooking temperature and the limited cooking time, the complete gelatinization of the granules prevent the use of damaged starch particles in the experiment. For assessing the behaviour of partly gelatinized starches in the embedding within the calculus matrix, it would be sufficient to use simple precautions during their cooking; these would involve a lower temperature

and a shorter cooking process. In addition, it would be also worth observing under the microscope the processed state of the molecules before inoculating the solution to the oral biofilm samples. In fact, slightly damaged granules are deemed to be more fragile and prone to further deterioration, thus resulting in a lower degree of inclusion in dental calculus (García-Granero, 2020).

A further step toward a more complete understanding of the embedding of food particles in dental calculus could be reached through the *in vitro* simulation of depositional and post-depositional processes; indeed, as previously claimed in this thesis, both the human and natural influence can cause taphonomic changes in dental calculus and it can result in analysing a biased material. It would be interesting to assess how much these processes can actually damage and modify dental calculus and to which level does it occur: would be possible to damage only the surface of the dental calculus and thus result in a slight alteration, or the mineralized plaque would be affected also on the internal structure?

A potential direction for future studies related to a better understanding of dental calculus as a material, may be an improvement of the protocol followed during this study, with the aim of producing an *in vitro* mineralized plaque as close as possible to the natural material. In order to obtain this result, it can be advisable to incorporate the alpha-amylase from the beginning of the experiment, so that the oral bacteria can come in contact with the enzyme sooner, as it happens *in vivo*. To better reflect the normal condition of the starch degradation in the oral environment, the refreshment of alpha-amylase should be carried out every two days in order to maintain a constant process of starch degradation within the biofilm model. These improvements may enable the use of the *in vitro* dental calculus for academic and research purposes, which can be extended to the study of other food microfossils such as phytoliths, thus allowing to conduct analysis of archaeological specimens only when strictly required and avoid destructive investigations on ancient evidence.

As previously stated, archaeological dental calculus investigation suffers from the poor knowledge which nowadays we still have of the material itself. Nevertheless, the vast majority of the study focused on the mineralized plaque are mostly interested in inferring new insights into the archaeological populations, while really few scholars focused on obtaining new information about the mechanisms which lead the food microfossils to get embedded into the calculus matrix. In order to avoid this tendency to produce an increasing number of biased or misleading archaeological information, it would be

desirable to encourage the research toward a deep understanding of both the dietary potential and the nature of dental calculus as a material.

6.3 Conclusions

Today, the analysis of dental calculus from the archaeological contexts proves to be one of the few direct evidence of food consumption preserved to the present day. While the academic world seems to be increasingly attracted to the study of this material with the aim of gain information on past dietary habits, little attention is paid to the fact that the mineralized plaque and the mechanisms which lead the food microfossils to be embedded in it are not fully understood. With the purpose of investigating this process, the present study focused on an oral biofilm model in order to remove as much as possible the influence of the external factors and be able to control the variables applied to the biofilm itself.

As demonstrated in the discussion chapter, the inclusion of starch granules in dental calculus is not the result of a stochastic process; instead, the physical characteristics of each species appear to play a fundamental role in the process itself. In fact, consistent results were obtained from the counts of starch granules embedded in the mineralized oral biofilm, showing a clear repetitive pattern in the inclusion of the food particles on the basis of their species and their size. Furthermore, alpha-amylase, the enzyme which is responsible for the first digestion of starch granules in the oral cavity, showed to perform a major function by degrading a high percentage of the food molecules available and thus preventing their embedding in the calculus matrix.

In conclusion, this study was not able to fully provide an explanation for the mechanism of starch granules inclusion in dental calculus but nevertheless, it allowed us to gain insights into the different behaviours undertaken by potato and wheat starch granules when they enter the oral environment. In order to deepen and explore this particular process, further research is needed, involving different starch species and other food microfossils. In addition, with the purpose of providing new information on dental calculus as a material, it would be recommended to further develop the method here employed. Diet is among the strongest forms of cultural differentiation that have ever existed, and it is our job as scientist to make the most out of it.

English Abstract

In the last fifteen years, the investigation of archaeological dental calculus has gained considerable interest among the academic world thanks to its ability of preserving fossilised evidence of the human past within its composite matrix. In particular, the potential of this material of providing insights into the dietary habits of past populations through the study of plant microfossils, resulted in intensive destructive analyses of this archaeological remain. Nonetheless, the mechanisms which lead the food particles to get embedded in the mineralized dental plaque are currently not fully understood, and thus the interpretation of this material may bring to misleading or biased outcomes. In recent years, although several progresses have been made on the developing of new non-destructive technique for the analysis of dental calculus, very few are the new advancements which try to investigate the processes and the pathways followed by the food molecules in the inclusion within the calculus matrix.

Throughout the development of an oral biofilm model, this study wants to analyse the behaviour of potato and wheat starch granules when exposed to the oral environment. Thanks to the *in vitro* setting, this study enables a high degree of control over the variables by which the biofilm, and thus the starches, are dependent; namely, in order to assess the potential of starch granules to represent dietary information when extracted from dental calculus, the role of alpha-amylase and the behaviour of different starch types was observed. The starch degradation carried out by the enzyme constituted a really important factor in the inclusion of the granules within the calculus matrix, by strongly decreasing the number of molecules available in the oral cavity. Similarly, the influence of the different starch granule types resulted in great discriminative factor in their embedding in the mineralized plaque. As this study was not fully able to provide explanation on the process of incorporation, further research is encouraged with the aim of obtaining a clear picture of dental calculus as evidence of past human diet.

Dutch Abstract

In de afgelopen vijftien jaar heeft het onderzoek van archeologisch tandsteen veel belangstelling gewekt in de academische wereld dankzij de mogelijkheid die het biedt om gefossiliseerd bewijs van het menselijk verleden te bewaren in zijn samenstelling. Vooral de potentie van dit materiaal om inzicht te verschaffen in de voedingsgewoonten van de vroegere mens door de studie van plantaardige microfossielen, heeft geleid tot intensieve destructieve analyses van dit archeologisch materiaal. Niettemin zijn de mechanismen die ervoor zorgen dat voedseldeeltjes in de gemineraliseerde tandplak ingebed raken, nog niet volledig begrepen. Als gevolg hiervan kan dit materiaal verkeerd worden geïnterpreteerd, met misleidende of vertekende resultaten als gevolg. De laatste jaren is er weliswaar vooruitgang geboekt bij de ontwikkeling van nieuwe niet-destructieve technieken voor de analyse van tandsteen, maar er zijn weinig nieuwe ontwikkelingen met betrekking tot het onderzoek naar de processen en paden die door de voedselmoleculen worden gevolgd bij de insluiting in de tandsteenmatrix.

Door middel van de ontwikkeling van een oraal biofilmmodel analyseert deze studie het gedrag van aardappel- en tarwezetmeelkorrels bij blootstelling aan de orale omgeving. Dankzij de *in vitro* setting maakt deze studie een hoge mate van controle mogelijk over de variabelen waarvan de biofilm, en dus de zetmelen, afhankelijk zijn. Om het potentieel van zetmeelkorrels te kunnen analyseren met betrekking tot dieet, wordt de rol van alpha-amylase en het gedrag van verschillende zetmeeltypes geobserveerd. De afbraak van zetmeel door het enzym vormde een zeer belangrijke factor bij de opname van de korrels in de tandsteenmatrix, doordat het aantal in de mondholte beschikbare moleculen sterk vermindert. Evenzo resulteert de invloed van de verschillende typen zetmeelkorrels in een grote discriminerende factor bij hun inbedding in de gemineraliseerde plaque. Aangezien deze studie geen volledige verklaring kan geven over het proces van inbedding, wordt verder onderzoek aangemoedigd met het oog op het verkrijgen van een duidelijk beeld van tandsteen als bewijs van historische voedingsgebruiken van de mens.

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List of Figures

List of Tables

7. Appendices

Appendix A: Results and set-up of the alpha-amylase activity assay.

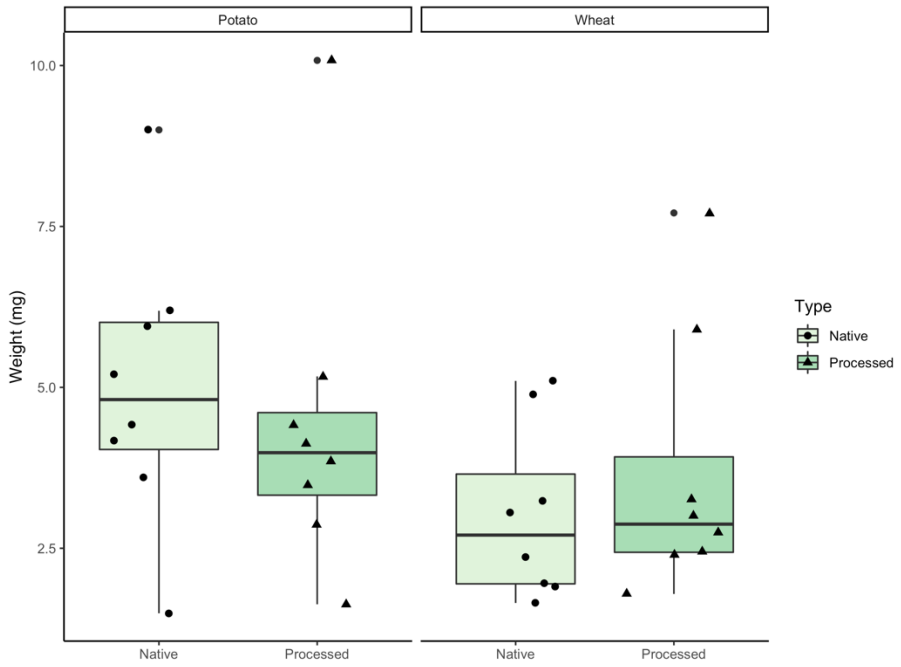
Plate	Well	Treatment	Wavelength	Absorbance	Row	Column	Activity
Plate 1	A01	Potato 1/2	540	135	A	1	0
Plate 1	A02	Potato 2/2	540	146	A	2	0
Plate 1	B01	Potato 1/2	540	172	B	1	0
Plate 1	B02	Potato 2/2	540	143	B	2	0
Plate 1	C01	Potato 1/2	540	503	C	1	2.57
Plate 1	C02	Potato 2/2	540	0.48	C	2	2.33
Plate 1	D01	Potato 1/2	540	161	D	1	0
Plate 1	D02	Potato 2/2	540	136	D	2	0
Plate 1	E01	Potato 1/2	540	208	E	1	0
Plate 1	E02	Potato 2/2	540	157	E	2	0
Plate 1	F01	Potato 1/2	540	0.17	F	1	0
Plate 1	F02	Potato 2/2	540	151	F	2	0
Plate 1	G01	Potato 1/2	540	179	G	1	0
Plate 1	G02	Potato 2/2	540	159	G	2	0
Plate 1	H01	Potato 1/2	540	145	H	1	0
Plate 1	H02	Potato 2/2	540	163	H	2	0
Plate 1	A03	Potato 1/2	540	162	A	3	0
Plate 1	A04	Potato 2/2	540	172	A	4	0
Plate 1	B03	Potato 1/2	540	177	B	3	0
Plate 1	B04	Potato 2/2	540	151	B	4	0
Plate 1	C03	Potato 1/2	540	419	C	3	1.7
Plate 1	C04	Potato 2/2	540	236	C	4	0
Plate 1	D03	Potato 1/2	540	186	D	3	0
Plate 1	D04	Potato 2/2	540	185	D	4	0
Plate 1	E03	Potato 1/2	540	155	E	3	0
Plate 1	E04	Potato 2/2	540	145	E	4	0
Plate 1	F03	Potato 1/2	540	807	F	3	5.73
Plate 1	F04	Potato 2/2	540	812	F	4	5.78
Plate 1	G03	Potato 1/2	540	243	G	3	0
Plate 1	G04	Potato 2/2	540	228	G	4	0
Plate 1	H03	Potato 1/2	540	181	H	3	0
Plate 1	H04	Potato 2/2	540	0.18	H	4	0

Plate 1	A05	Potato 1/2	540	126	A	5	0
Plate 1	A06	Potato 2/2	540	268	A	6	0
Plate 1	B05	Potato 1/2	540	151	B	5	0
Plate 1	B06	Potato 2/2	540	92	B	6	0
Plate 1	C05	Potato 1/2	540	301	C	5	0
Plate 1	C06	Potato 2/2	540	324	C	6	0
Plate 1	D05	Potato 1/2	540	142	D	5	0
Plate 1	D06	Potato 2/2	540	139	D	6	0
Plate 1	E05	Potato 1/2	540	173	E	5	0
Plate 1	E06	Potato 2/2	540	176	E	6	0
Plate 1	F05	Potato 1/2	540	247	F	5	0
Plate 1	F06	Potato 2/2	540	234	F	6	0
Plate 1	G05	Potato 1/2	540	155	G	5	0
Plate 1	G06	Potato 2/2	540	154	G	6	0
Plate 1	H05	Potato 1/2	540	0.14	H	5	0
Plate 1	H06	Potato 2/2	540	137	H	6	0
Plate 1	A07	Potato 1/2	540	145	A	7	0
Plate 1	A08	Potato 2/2	540	188	A	8	0
Plate 1	B07	Potato 1/2	540	601	B	7	3.59
Plate 1	B08	Potato 2/2	540	676	B	8	4.37
Plate 1	C07	Potato 1/2	540	0.75	C	7	5.14
Plate 1	C08	Potato 2/2	540	713	C	8	4.75
Plate 1	D07	Potato 1/2	540	138	D	7	0
Plate 1	D08	Potato 2/2	540	151	D	8	0
Plate 1	E07	Potato 1/2	540	186	E	7	0
Plate 1	E08	Potato 2/2	540	197	E	8	0
Plate 1	F07	Potato 1/2	540	705	F	7	4.67
Plate 1	F08	Potato 2/2	540	711	F	8	4.73
Plate 1	G07	Potato 1/2	540	664	G	7	4.24
Plate 1	G08	Potato 2/2	540	671	G	8	4.32
Plate 1	H07	Potato 1/2	540	162	H	7	0
Plate 1	H08	Potato 2/2	540	0.16	H	8	0
Plate 2	A03	wheat 1/2	540	146	A	3	0
Plate 2	A04	wheat 2/2	540	0.15	A	4	0
Plate 2	B03	wheat 1/2	540	129	B	3	0
Plate 2	B04	wheat 2/2	540	144	B	4	0
Plate 2	C03	wheat 1/2	540	177	C	3	0
Plate 2	C04	wheat 2/2	540	177	C	4	0
Plate 2	D03	wheat 1/2	540	173	D	3	0
Plate 2	D04	wheat 2/2	540	174	D	4	0
Plate 2	E03	wheat 1/2	540	136	E	3	0

Plate 2	E04	wheat 2/2	540	137	E	4	0
Plate 2	F03	wheat 1/2	540	449	F	3	2.01
Plate 2	F04	wheat 2/2	540	437	F	4	1.88
Plate 2	G03	wheat 1/2	540	178	G	3	0
Plate 2	G04	wheat 2/2	540	178	G	4	0
Plate 2	H03	wheat 1/2	540	172	H	3	0
Plate 2	H04	wheat 2/2	540	158	H	4	0
Plate 2	A01	wheat 1/2	540	0.15	A	1	0
Plate 2	A02	wheat 2/2	540	141	A	2	0
Plate 2	B01	wheat 1/2	540	138	B	1	0
Plate 2	B02	wheat 2/2	540	131	B	2	0
Plate 2	C01	wheat 1/2	540	313	C	1	0
Plate 2	C02	wheat 2/2	540	287	C	2	0
Plate 2	D01	wheat 1/2	540	151	D	1	0
Plate 2	D02	wheat 2/2	540	136	D	2	0
Plate 2	E01	wheat 1/2	540	169	E	1	0
Plate 2	E02	wheat 2/2	540	157	E	2	0
Plate 2	F01	wheat 1/2	540	155	F	1	0
Plate 2	F02	wheat 2/2	540	151	F	2	0
Plate 2	G01	wheat 1/2	540	139	G	1	0
Plate 2	G02	wheat 2/2	540	143	G	2	0
Plate 2	H01	wheat 1/2	540	0.14	H	1	0
Plate 2	H02	wheat 2/2	540	138	H	2	0
Plate 2	A05	wheat 1/2	540	209	A	5	0
Plate 2	A06	wheat 2/2	540	0.1	A	6	0
Plate 2	B05	wheat 1/2	540	119	B	5	0
Plate 2	B06	wheat 2/2	540	111	B	6	0
Plate 2	C05	wheat 1/2	540	225	C	5	0
Plate 2	C06	wheat 2/2	540	238	C	6	0
Plate 2	D05	wheat 1/2	540	118	D	5	0
Plate 2	D06	wheat 2/2	540	116	D	6	0
Plate 2	E05	wheat 1/2	540	156	E	5	0
Plate 2	E06	wheat 2/2	540	154	E	6	0
Plate 2	F05	wheat 1/2	540	186	F	5	0
Plate 2	F06	wheat 2/2	540	185	F	6	0
Plate 2	G05	wheat 1/2	540	123	G	5	0
Plate 2	G06	wheat 2/2	540	126	G	6	0
Plate 2	H05	wheat 1/2	540	142	H	5	0
Plate 2	H06	wheat 2/2	540	124	H	6	0
Plate 2	A07	wheat 1/2	540	151	A	7	0
Plate 2	A08	wheat 2/2	540	147	A	8	0

Plate 2	B07	wheat 1/2	540	0.47	B	7	2.23
Plate 2	B08	wheat 2/2	540	437	B	8	1.88
Plate 2	C07	wheat 1/2	540	484	C	7	2.37
Plate 2	C08	wheat 2/2	540	483	C	8	2.36
Plate 2	D07	wheat 1/2	540	0.13	D	7	0
Plate 2	D08	wheat 2/2	540	128	D	8	0
Plate 2	E07	wheat 1/2	540	176	E	7	0
Plate 2	E08	wheat 2/2	540	157	E	8	0
Plate 2	F07	wheat 1/2	540	488	F	7	2.24
Plate 2	F08	wheat 2/2	540	521	F	8	2.76
Plate 2	G07	wheat 1/2	540	503	G	7	2.57
Plate 2	G08	wheat 2/2	540	513	G	8	2.67
Plate 2	H07	wheat 1/2	540	149	H	7	0
Plate 2	H08	wheat 2/2	540	0.14	H	8	0

Appendix B: Dry-weight in mg of the samples divided for starch granule type and processed and native starches.



Appendix C: Mean corrected count of starch granules from the samples with standard deviation, grouped by different sizes: small (>10µm), medium (10-20µm) and large (<20µm).

	Amylase	Small (%)	SD	Medium (%)	SD	Large (%)	SD	Total (%)
Potato	Present	193 (46.17%)	293	209 (50%)	238	16 (3.8%)	27	418 (100%)
Potato	Absent	494 (20.78%)	138	1594 (67.05%)	478	289 (12.15%)	226	2377 (100%)
Wheat	Present	0	0	0	0	0	0	0
Wheat	Absent	22136 (96.83%)	9956	688 (3%)	237	36 (0.15%)	72	22860 (100%)