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Bjørn Peare Bartholdy Amanda G. Henry

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Abstract

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1 Introduction

Dental calculus, mineralised dental plaque, has proven to contain a wealth of dietary information in the form of plant microfossils (Hardy et al., 2009; Henry & Piperno, 2008), proteins (Hendy et al., 2018; Warinner, Hendy, et al., 2014), and other organic residues (Buckley et al., 2014). It has also proven to be able to retain and preserve dietary information for millenea, providing detailed dietary information on past populations (Henry & Piperno, 2008; Jovanović et al., 2021; Tao et al., 2020) and species (Hardy et al., 2012; Henry et al., 2014).

Until recently, few studies directly investigated the presence of plant microremains in the dental calculus of archaeological remains. The ability to extract phytoliths from the dental calculus of archaeological fauna to investigate diet was first noted by Armitage (1975), and later by Middleton and Rovner (1994), and Fox and colleagues (1996). The ability to detect coca consumption by looking at human dental calculus was explored by Klepinger and Kuhn (1977), and starches and phytoliths were extracted from human dental calculus by Cummings and Magennis (1997).

In more recent years, the study of dental calculus has increased exponentially, and the wealth of information contained within the mineralised matrix has been, to a larger extent, acknowledged. The applications of

dental calculus span a wide variety of archaeological research areas, such as oral microbiome characterisation (including pathogens) through the analysis of DNA and proteins (Adler et al., 2013; Warinner, Rodrigues, et al., 2014), microbotanical remains (Hardy et al., 2009; Henry & Piperno, 2008; Mickleburgh & Pagán-Jiménez, 2012), other organic residues and proteins from dietary compounds (Buckley et al., 2014; Hendy et al., 2018), and nicotine-use (Eerkens et al., 2018). Especially the extraction of starch granules has become a rich source of dietary information, as starch granules have proven to preserve well within dental calculus over a variety of geographical and temporal ranges (Henry et al., 2014; Jovanović et al., 2021; Piperno & Dillehay, 2008; Tao et al., 2020).

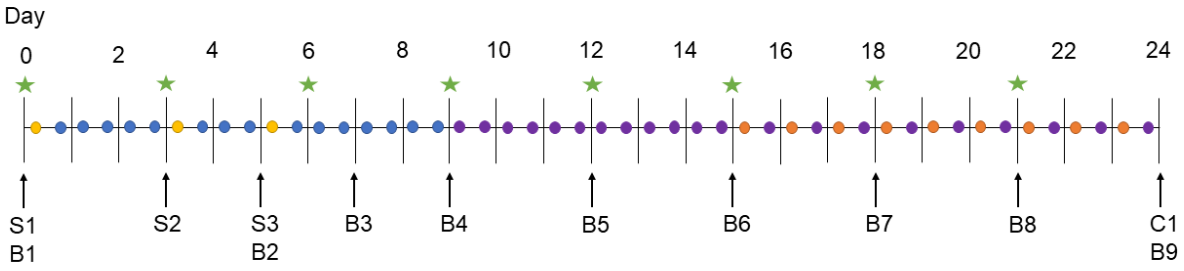
Despite this, our knowledge of dental calculus and the incorporation pathways of the various markers is limited (Radini et al., 2017), as is our knowledge of information-loss caused by these pathways and the methods we use to extract and analyse dental calculus and make inferences on past diets. Extraction methods were tested by Tromp and colleagues (2017), specifically regarding decalcification using HCl or EDTA. The authors found significantly more starches with the EDTA extraction method than the HCl extraction method; however, as noted by the authors, comparisons involving archaeological calculus are problematic due to variability between and within individuals. Other studies have been conducted on modern populations (Leonard et al., 2015) and primates (Power et al., 2015), both with well-documented dietary intake, to reconcile our interpretations on the recovery of microremains (both phytoliths and starches) from dental calculus and how well they represent actual dietary intake. These studies are justifiably limited, despite meticulous documentation and observation, due to unknown variables and uncertainty involved when studying living organisms. Dental calculus is a complex oral biofilm with a multifactorial aetiology and variable formation rates both within and between individuals (Jepsen et al., 2011), contributing to the stochasticity of starch representation being observed in numerous studies. Additionally, the rate of α-amylase differs both between and within individuals (Froehlich et al., 1987; Nater et al., 2005), causing different rates of hydrolysis of the starch granules present in the oral cavity. Add to this the effects of the many different methods of starch processing, as well as post-depositional processes that are still being explored (García-Granero, 2020), and you have a highly unpredictable process.

In this exploratory study, we use an oral biofilm model to investigate the retention of starch granules within

dental calculus in a controlled laboratory setting, allowing us full control over dietary input. We use a multiwell setup for consistent, high-throughout analysis. Our main questions concern the representation of granules extracted from the calculus compared to the actual intake. How much of the original diet is incorporated into the calculus, and how much is recovered? Is there differential loss of information from specific dietary markers that affects the obtained dietary information, and how does this affect the representation of diet from extracted microremains?

We find that, despite an absence of α-amylase in the model, a limited proportion of the starch input is actually retained in the calculus, as well as a shift in the size ratios of individual starch granules that are incoporated into the calculus. We also show that the number of starch granules that are incorporated increases as the size of the calculus deposit increases.

2 Materials and Methods



S = saliva samples
B = artificial saliva samples
B1 = artificial saliva stock solution
C = final plaque sample

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

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

2.1 Biofilm formation

In this study we employ a multispecies oral biofilm model following a modified protocol from Sissons and colleagues (1991) and Shellis (1978). The setup comprises a polypropylene 24 deepwell PCR plate (KingFisher 97003510) with a lid containing 24 pegs (substrata), which is autoclaved at 120°C, 1 bar overpressure, for 20 mins.

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

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

Starch treatments were initiated on day 9 to avoid starch granule counts being affected by α-amylase hydrolysis. An α-amylase (EC 3.2.1.1) activity assay was conducted to confirm that no amylase was present in the system before starch treatments started. Starch treatments replaced sucrose treatments, occurring twice

per day for six minutes. The starch treatments involved transferring the substrata to a new plate containing a 0.25% (w/v) starch from potato (Roth 9441.1) solution, a 0.25% (w/v) starch from wheat (Sigma S5127) solution, and a 0.5% (w/v) mixture of equal concentrations (w/v) wheat and potato. All starch solutions were created in a 5% (w/v) sucrose solution. Before transferring biofilm samples to the starch treatment plate, the plates were agitated to keep the starches in suspension in the solutions. During treatments, the rpm was increased to 60 to facilitate contact between starch granules and biofilms.

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

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

2.2 Amylase activity detection

An α-amylase (EC 3.2.1.1) activity assay was conducted on artificial saliva samples collected from the plate wells on days 3, 5, 6, 9, and 12. Whole saliva samples were collected on days 0, 3, and 5 as positive controls. Collected samples were stored at 4°C until the assay was conducted on day 18. All samples and standard curves were run in triplicates on two separate plates. Positive control saliva samples were compared against a standard curve containing H_2O, while artificial saliva samples were compared against a standard curve containing sterile artificial saliva (due to the colour of artificial saliva). Two photometric readings were conducted for each plate with a 540 nm filter on a… The protocol is a slightly modified version of an Enzymatic Assay of α-Amylase (https://www.sigmaaldrich.com/NL/en/technical-documents/protocol/protein-biology/enzyme-

activity-assays/enzymatic-assay-of-a-amylase) (Bernfeld, 1955), which measures the amount of maltose released from starch by α-amylase activity. Results are reported in units (U) per mL enzyme, where 1 U releases 1 mg of maltose. For a more detailed protocol, see

2.3 Starch counts

Starch granule counts were divided into three size categories: small (<10 μm), medium (10 – 20 μm), and large (>20 μm). Counts for small wheat and potato starches were combined for the mixed-treatment samples, as it was not possible to distinguish betweem the small starch granules from the two species.

2.3.1 Treatment solutions

A 1 ml aliquot of each starch solution was taken, from which 10 μl was mounted on a microscope slide with an 18 x 18 mm coverslip, and counted under a light microscope (Zeiss Axioscope A1). Counting starches on a full slide was not feasible, so three slide transects were counted (at ca. 1/4, 1/2, and 3/4 of the slide), and the sample counts were extrapolated (see Supplementary material for more details).

2.3.2 Extraction method

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

2.4 Statistical analysis

Statistical analysis was conducted in R version 4.1.1 (2021-08-10) (R Core Team, 2020). A one-way ANOVA with sample weight as the dependent variable (DV) and treatment as the grouping variable (GV) was

conducted to explore the effect of the different starch treatments on biofilm growth.

To calculate size proportions, mean counts for each treatment were taken across both experimental plates for each treatment, resulting in a mean count for each granule size category within each treatment.

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

All scripts and data used in the analysis are available on OSF (https://osf.io/uc5qy/) and Github (https://github.com/bbartholdy/byoc-starch). All protocols are available on protocols.io.

3 Results

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

3.1 No amylase activity detected in the model

No appreciable amount of α-amylase activity was detected in any of the artificial saliva samples from any of the days that were sampled. Only positive controls contained amylase activity that could be detected in the assay (Table 1). The results from the additional plates and photometric readings can be found in the Supplementary Materials. The results are not comparable to other studies presenting α-amylase activity levels in humans; however, they are sufficient to show that there is no activity in the system.

Table 1: Amylase activity in U/mL enzyme, where a U is mg maltose released from starch in six minutes at 36 degrees Celsius. Plate 1, photometric reading 1. Negative values converted to 0.

	1	2	3
S1	9.66	3.44	9.74
S2	10.30	4.75	9.61
S3	9.19	5.15	9.67
B1	0.00	0.00	0.00
B2	0.00	0.00	0.00
B3	0.00	0.00	0.00
B4	0.00	0.00	0.00
B5	0.00	0.00	0.00
BT1	0.00	0.00	0.00
BT2	0.00	0.00	0.00
BT3	0.00	0.00	0.00

Table 2: Summary statistics for biofilm dry-weights (in mg) by treatment.

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

3.2 Treatment type had no effect on biofilm growth

A one-way ANOVA suggests that the type of starch used during the biofilm growth period had a minimal effect on the growth of the biofilm (expressed as total dry weight of the sample), $F(3, 43) = 1.16$, $p = 0.335$. A summary of sample weights is available in Table 2.

3.3 Starch counts

It was not possible to differentiate between potato and wheat starches smaller than ca. 10 μm, small potato starches were counted as wheat starches in the mixed-treatment samples. This is reasonable given that

Table 3: Mean starch counts from solutions, including the proportional makeup of the different sizes of granules.

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

Table 4: Mean starch counts extracted from samples with standard deviation (SD), including the proportion of granule sizes of the total count.

Treatment	Starch	Small (%)	SD	Medium (%)	SD	Large (%)	SD	Total (%)	SD
mix	potato	NaN (NA)	NA	1959 (79.6%)	1800	501 (20.40%)	446	2460 (100%)	2190
mix	wheat	9515 (54.60%)	8860	6522 (37.4%)	6030	1381 (7.93%)	1200	17417 (100%)	15900
mix	both	9515 (47.90%)	8860	8480 (42.7%)	7650	1882 (9.47%)	1600	19877 (100%)	17800
potato	potato	351 (7.24%)	297	3565 (73.6%)	2400	930 (19.20%)	929	4846 (100%)	3320
wheat	wheat	15235 (55.00%)	11900	12148 (43.9%)	11100	1953 (7.06%)	2020	27680 (100%)	23600

Table 5: The mean percentage of starches from the solutions that were incorported into the samples.

Treatment	Starch	Small	Medium	Large	Total
mix	potato	NA	0.186%	0.054%	0.124%
mix	wheat	0.051%	0.102%	0.077%	0.064%
mix	both	0.051%	0.114%	0.069%	0.069%
potato	potato	0.284%	0.266%	0.060%	0.161%
wheat	wheat	0.094%	0.189%	0.069%	0.109%

the small potato granules make up an insignificant proportion of the total count of small granules within mixed-treatment solutions, which are predominantly wheat granules (99.2%).

The separate wheat and potato solutions were made with a 0.25% (w/v) starch concentration, while the mixed-starch solution was made with 0.25% (w/v) of each starch, with a total concentration of 0.50% (w/v). The mixed treatment had the highest absolute count of starch granules in solution (mean = 1.9797333×10^6, 2.7035733×10^7), while the biofilms exposed to the wheat solution preserved the greatest number of granules (mean = 2.7679545×10^4). The potato treatment had the lowest absolute counts in both the solution (3.016×10^6) and in the biofilm samples (4845.9090909) (Tables 3 and 4).

3.3.1 Proportion of available starches incorporated in samples

The proportion of total starches from the solutions that were incorporated into the samples ranged from 0.064% to 0.161%, with potato granules being more readily incorporated than wheat in both the separated- and mixed-treatment samples (Table 5). There is an inverse relationship between the absolute starch count in the solutions and the proportional incorporation of starches in the biofilm samples, i.e. potato had the lowest absolute count in solutions, but the highest proportional incorporation, and vice versa for the mixed treatment.

Wheat incorporation was most affected in the mixed-treatment samples, with only 0.064% of the total available starches being incorporated into the sample, compared to 0.161% in the separated wheat treatment.

3.3.2 Size ratios differ between solutions and samples

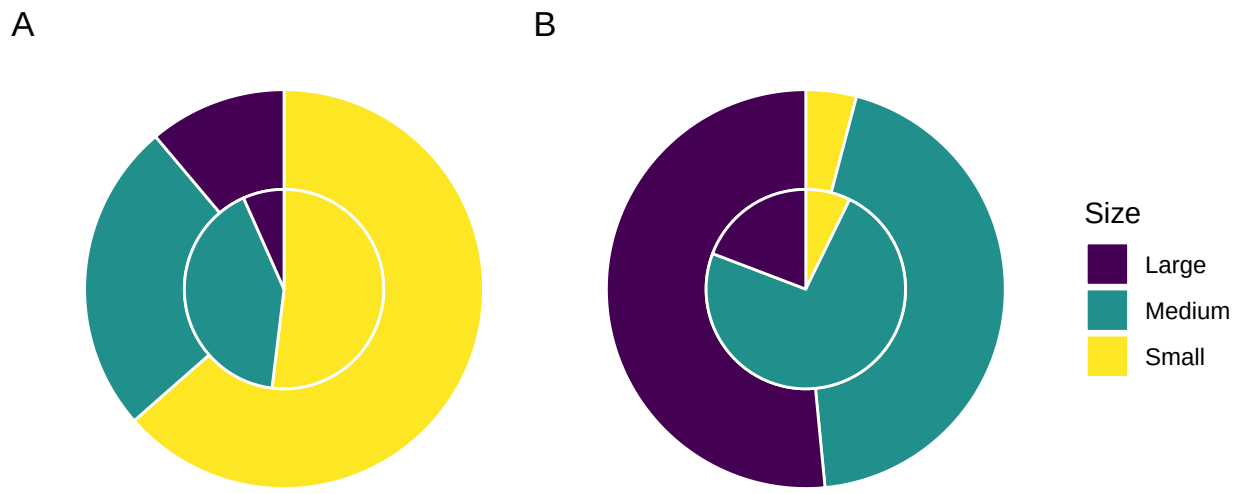


Figure 1: (A) Proportion (%) of sizes of starch granules in the wheat solution (outer ring) and extracted from the wheat-treatment samples (inner ring), and (B) in the potato solution (outer ring) and extracted from the potato-treatment samples (inner ring).

Overall, medium starch granules had a higher mean rate of incorporation (0.186%) than small (0.143%) and large (0.065%) starch granules across all treatments, while large potato starches had the lowest rate of incorporation across all treatments.

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

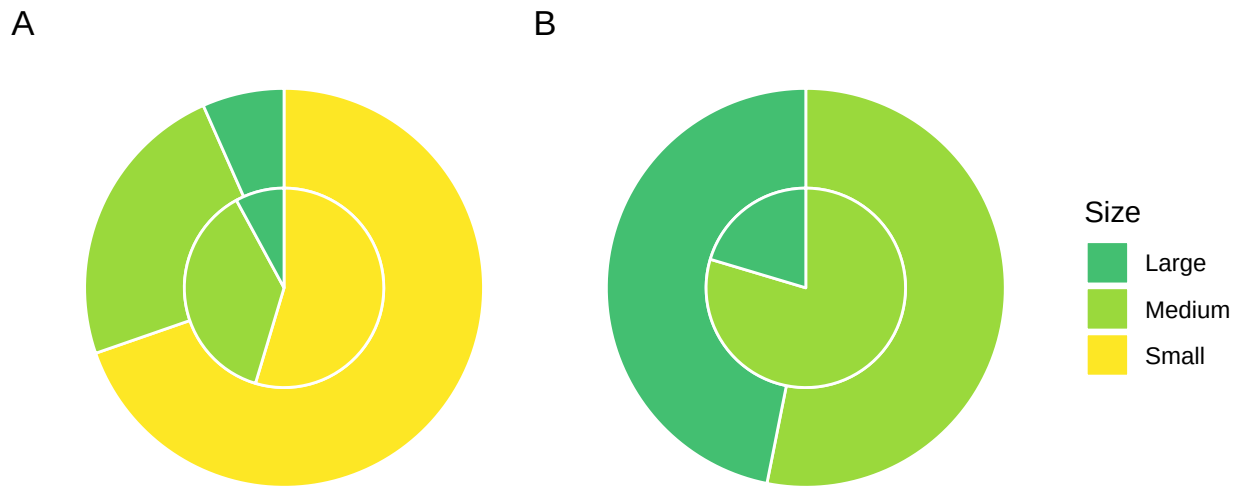


Figure 2: Proportion of sizes of (A) wheat granules in the mixed solution (outer ring) and extracted from the wheat-treatment samples (inner ring), and sizes of (B) potato granules in the solution (outer ring) and extracted from the potato-treatment samples (inner ring).

in wheat treatments and increased in potato treatments (Figures 1 and 2).

3.3.3 Biofilm weight correlated positively with extracted starch counts

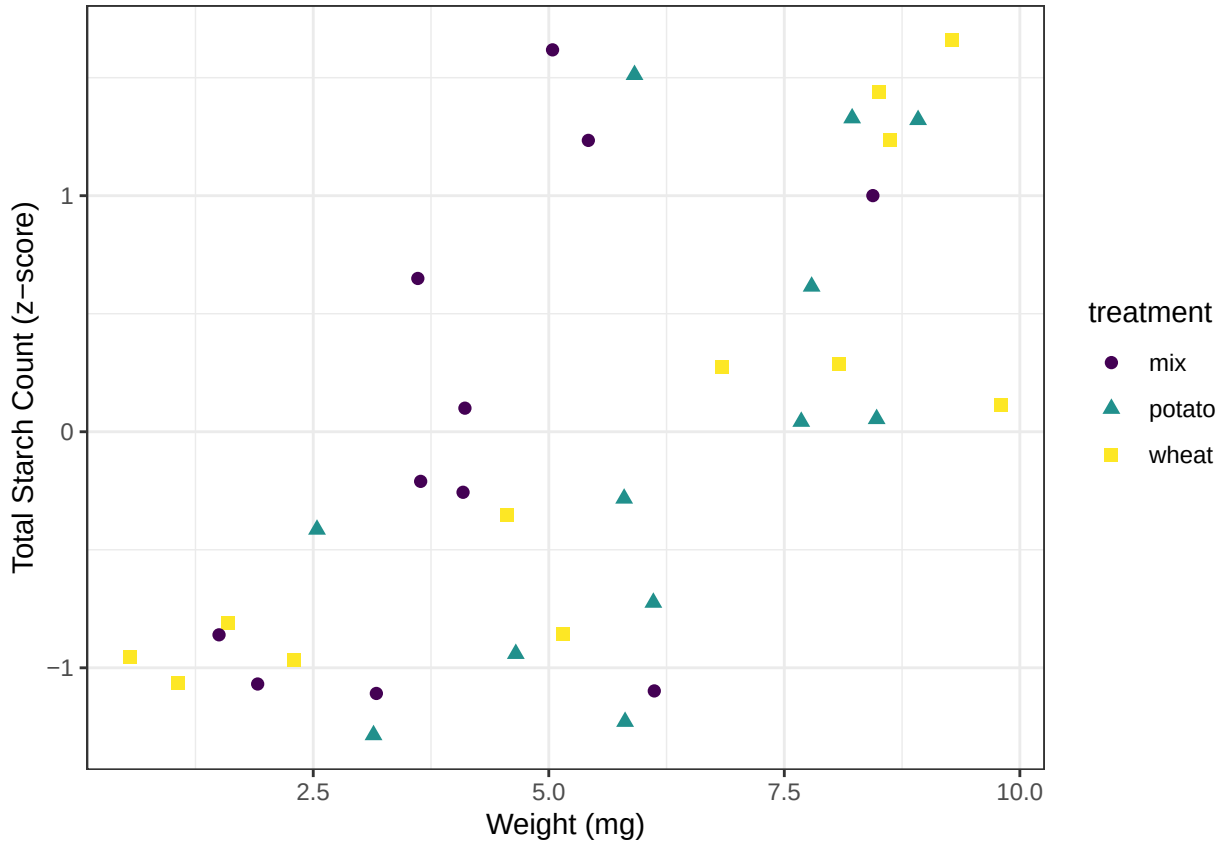


Figure 3: Scatter plot of sample weight and standardised starch count by Z-score for seprated treatments.

Pearson's r suggests a strong positive correlation between the total weight of the biofilms and the total starch count (standardised by z-score) extracted from the samples across treatments, $r = 0.659$, 90%CI[0.463, 0.794], p = < 0.001 (Figure 3).

The same test was applied to total biofilm weight and starch count per mg calculus (also standardised by z-score), resulting in a weak positive correlation, $r = 0.3$, 90%CI[0.0618, 0.506], p 0.0403 (Figure 4).

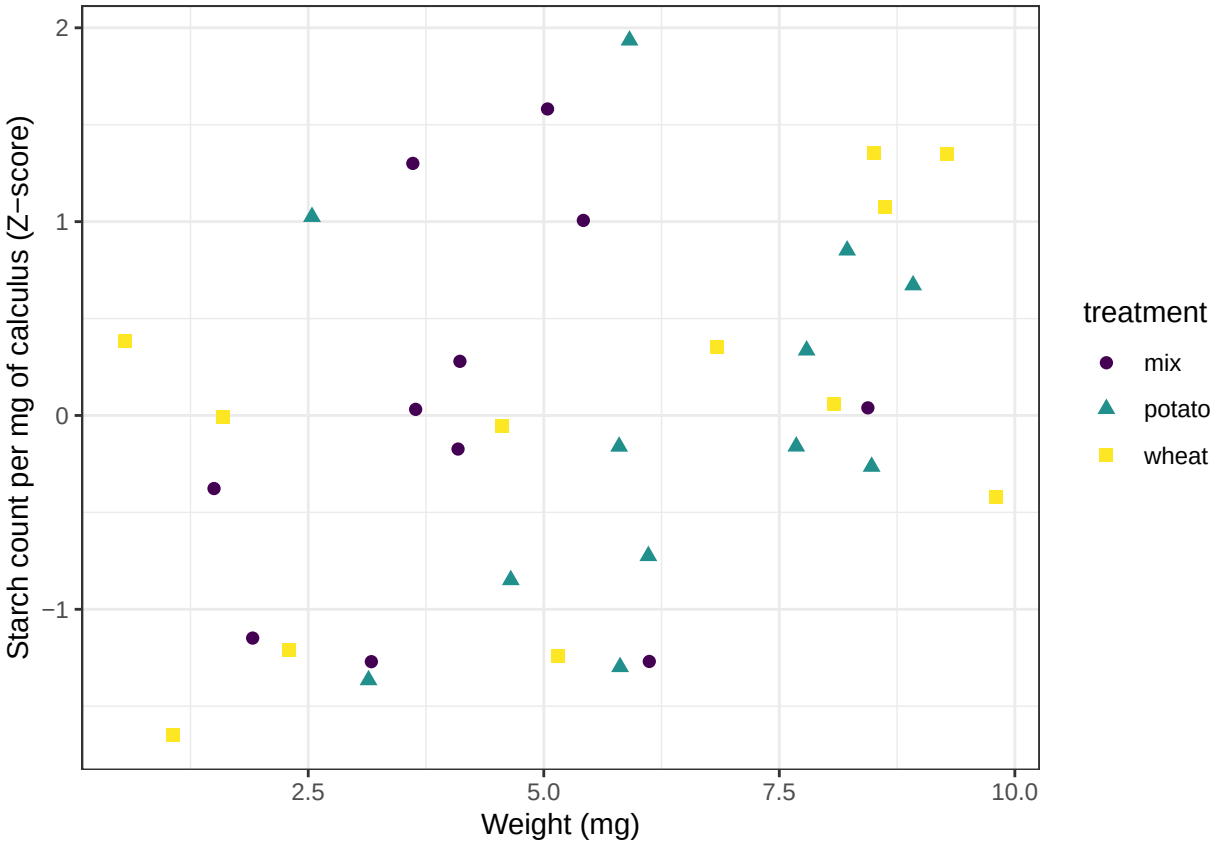


Figure 4: Scatter plot of sample weight in mg and standardised count of starch grains per mg calculus.

4 Discussion

Here, we have provided a method for exploring the incorporation of dietary starches into the mineral matrix of a dental calculus biofilm model. Our results show that a very low proportion of the starches exposed to the biofilm during growth are retained in the mineral matrix, and that the size of the starch granules may affect the likeliness of incorporation. The proportions of starch granules (of all sizes) present in the extracted samples were similar across all treatments (0.064% to 0.161%), despite large differences in absolute granule counts between wheat (25,404,000) and potato (3,016,000) solutions.

The absolute counts, however, differed more visibly between treatments and was proportional with the total count of granules in the treatment solutions. Wheat and mixed solutions had the highest absolute mean count of starch granules, and also had the highest absolute mean count of starch granules extracted from the dental calculus (Tables 3 and 4). This suggests that the starches that are more frequently consumed will be present in higher quantities in the dental calculus, at least prior to inhumation and degradation in the burial environment; although, starch availability is unlikely to be a main factor in starch incorporation. Despite the low proportion of granules recovered from the model calculus (0.064% to 0.161%), the absolute counts were still substantially greater than counts recovered from archaeological remains (Tromp et al., 2017; Tromp & Dudgeon, 2015; Wesolowski et al., 2010).

Previous research conducted on dental calculus from contemporary humans and non-human primates suggest a high level of stochasticity involved in the retention of starch granules in dental calculus, and that starch granules extracted from dental calculus are underrepresented with regard to actual starch intake, which is consistent with our findings (illustrated by high standard deviations and low proportional incorporation). Leonard and colleagues (2015) found individual calculus samples to be a poor predictor of diet in a population, as many of the consumed plants were missing from some individual samples, but were present in others. Power and colleagues (2015) presented similar findings in non-human primates, where phytoliths were more representative of individual diets than starch granules, and plants that produce predominantly larger starch (10-20 μm) granules were over-represented. Both studies concluded that the dietary breadth of a population can be captured in a sufficiently large sample, but interpretations on individual diets from microremains in

dental calculus is tenuous, at best (Leonard et al., 2015; Power et al., 2015).

We have also shown that the size of the starch granules influences the likelihood of incorporation into the calculus. Starch granules larger than 20 μm in diameter were underrepresented in the calculus samples compared to the original starch solutions, an effect that was consistent across all three treatments, while medium granules (10 – 20 μm) were often over-represented (Table 5, and Figures 1 and 2). This was especially true for the potato starches, which can reach up to 100 μm in diameter, whereas wheat generally only reach up to 35 μm (Gismondi et al., 2019; Haslam, 2004). This is consistent with the findings by Power and collagues (2015) mentioned previously, which found that the large (defined as medium in this study) granule-producing plants were overrepresented; although, the representation of granules larger than 20 μm in that study is unclear. Since those results were obtained on a modern population of chimps that were only buried for a short period of time, degradation was likely limited (Power et al., 2015). The taphonomy of starch granules has been addressed previously, and depends on burial environment (pH, temperature, microorganisms), as well as processing of the granules prior to deposition (García-Granero, 2020; Haslam, 2004; Henry et al., 2009). The potential for detrimental diagenetic effects on starch granules increases as the size and amylose content decreases (Franco et al., 1992; Haslam, 2004). Combined, the effect of intra-oral starch incorporation and the bias against large granules, together with the increased effect of taphonomy on small granules, would eliminate a large portion of the consumed starch granules, and could explain the large descrepency between our counts (unaffected by hydrolysis and taphonomy) and the aforementioned studies.

Another potentially important factor from our results is the size of the calculus deposit, which seems to influence the quantity of starches extracted from the calculus, as we found a strong positive correlation between biofilm size and retained starch granules (Figure 3); a result that contradicts findings from archaeological contexts (Dudgeon & Tromp, 2014; Wesolowski et al., 2010). When the concentration of starch granules per mg calculus is considered, the correlation is weaker, but still present (Figure 4); while the larger deposits contain a higher absolute count, our findings also suggest that they contain a slightly higher concentration of starches. The lack of a correlation in archaeological contexts could be due to diagenetic effects; or, the absence of amylase activity in our study could be impacting our interpretations.

The mechanism by which starch granules are incorporated into plaque and calculus remains largely unknown,

and few studies have directly investigated potential mechanisms. We know that a proportion of the starch granules entering the mouth can become trapped in the plaque/calculus, and can be recovered from archaeological samples of significant age (Buckley et al., 2014; Henry et al., 2014; Wu et al., 2021). Studies have also shown that not all starch granules come from a dietary source. Other pathways include cross-contamination from plant interactions in soil, such as palm phytoliths adhering to the skin of sweet potatoes (Tromp & Dudgeon, 2015), or accidental ingestion not related to food consumption (Radini et al., 2019; Radini et al., 2017).

When starch granules enter the mouth, whether through ingestion of food or accidental intake, they immediately encounter multiple obstacles. It is likely that the bulk of starch granules are swallowed along with the food, and are only briefly present in the oral cavity. Other granules that are broken off during mastication may be retained in the dentition. These granules are then susceptible to mechanical removal by the tongue, salivary clearance, and hydrolysis by α-amylase (Kashket et al., 1996). Starch granules that are trapped in crevices and channels on the surface of the mineralised plaque are (at least to some extent) protected from salivary clearance and mechanical cleaning actions of the tongue and lips, especially once a new layer of plaque has covered the surface of the calculus. This may explain why a previous study found starches more commonly in clusters, rather than dispersed over the surface of the dental calculus (Power et al., 2014). The authors hypothesised that the granules are either deposited in clusters, or group together in cracks and crevices within the mineral matrix. Unmineralised Lacunae and channels within the calculus matrix have been shown to contain viable bacteria, and may also be large enough to contain starch granules. These can range from the width of a single cell, to multiple-cell width (B. Tan et al., 2004), of which the latter could feasibly contain starch granules. If this is indeed one of the incorporation mechanisms of starch granules, then the limit of incorporated starches is set by the number and size of channels. Since this is likely controlled by the size of the calculus deposit, then the number of starches will increase as the size of the biofilm increases, which is consistent with our results (Figures **??** and 4). The size bias against large granules (>20 μm) may give further credence to the incorporation pathway of starch granules primarily in cracks and crevices in the calculus, as the smaller starch granules have an advantage over larger granules, and can be stored in larger quantities.

While potentially protected against clearance, granules trapped in plaque/calculus may still be susceptible to hydrolysis, as α-amylase has the ability to bind to both tooth enamel and bacteria within a biofilm and retain a portion of its hydrolytic activity (Nikitkova et al., 2013; Scannapieco et al., 1993; B. Tan et al., 2004; B. T. K. Tan et al., 2004). The susceptibility of granules to hydrolysis depends on the crystallinity and size of the starch granule, as well as the mode of processing. As mentioned previously, smaller starch granules are more susceptible to enzymatic degradation, as are pre-processed starches (e.g. by cooking), while dehydrated starches will have a reduced susceptibility (Björck et al., 1984; Franco et al., 1992; Haslam, 2004; Henry et al., 2009; Lingstrom et al., 1994). Given the lack of α-amylase activity detected in our model, hydrolysis is not the cause of the low incorporation rate. More details on the absence of α-amylase activity is discussed elsewhere (Bartholdy et al. in prep.). What we can say, is that the dietary picture we obtain from starch granules extracted from dental calculus reflects a number of individual, dietary, and environmental (both pre- and post-mortem environments) factors, and that this will likely be a somewhat random snapshot given the irregularity of plaque mineralisation within and between individuals (Jepsen et al., 2011; Jin & Yip, 2002). Although, certain factors may increase the likelihood of incorporation, such as abundance, size, and morphology of granules.

This article presents preliminary research exploring the potential of an oral biofilm model for dietary research in archaeology. A limitation of this study is the absence of α-amylase in the model. The presence of α-amylase will likely affect the total granule counts as well as the size ratios, as smaller starches may be more susceptible to hydrolysis (Franco et al., 1992; Haslam, 2004); however, the lack of α-amylase in the system is a beneficial side effect, as it can allow us to directly explore the effect of α-amylase on starch counts in future experiments, where α-amylase can be added to the model in concentrations similar to those found in the oral cavity (Scannapieco et al., 1993). While we are able to show the differing incorporation caused by absolute counts available, this was merely a side-effect of the difference in the number of granules in potato and wheat solutions of the same concentration (w/v). Further research should test multiple differing concentrations of the same starch type. The use of EDTA may also have affected counts. While previous studies have shown negligible morphological changes caused by exposure to EDTA (Le Moyne & Crowther, 2021; Modi et al., 2020; Tromp et al., 2017), these studies have not considered changes to separate size

categories within starch types, and whether shifts in size ratios occur due to exposure to the pre-treatment chemicals. The total number of granules on a slide often exceeded a number that was feasible to count in a reasonable time period, so we calculated the total counts by extrapolating from three slide transects. Thus, we reasonably assume that the three transects are a good representation of the entire slide, and that the distribution of all granules on the slide is relatively homogenous.

Finally, we only used native starches in the experimental procedure and the results will likely differ for processed starches (García-Granero, 2020). Based on counts obtained by Leonard and colleagues (2015, Supplement 2), processing and amylase may have a substantial effect on starch granule retention in the oral cavity.

The oral biofilm model described in this study, and in Bartholdy and colleagues (in prep.), provides a method to explore the incorporation and extraction of dietary compounds from dental calculus in a controlled laboratory setting, as many of the variables can be adjusted. The addition of known starch species in separate samples also removes the identification bias against the smaller undiagnostic starches. It can also address the call for more baseline testing of biases associated with dietary research conducted on dental calculus (Le Moyne & Crowther, 2021). *In vivo* studies on humans and non-human primates will be limited by the variability occurring between individuals, and difficulties in accurately documenting the entirety of starch granules entering the oral cavity at any given moment, especially accidental intake. Our experimental setup allows us a higher degree of control over the factors that influence starch incorporation, such as dietary intake, processing of starches leading to differential survivability, and inter- and intra-individual variation in plaque accumulation and mineralisation. The latter is especially difficult to control *in vivo* as it is influenced by numerous factors including genetics, diet, and tooth morphology (Jepsen et al., 2011). It can also facilitate training of students and researchers on methods of dental calculus analysis, such as starch and phytolith extraction and identification, where it can replace the use of finite archaeological resources.

5 Conclusions

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

While we have shown multiple factors that influence the likelihood of incorporation, the process still appears to be somewhat stochastic. Further research is needed to make sense of the contributing factors, and to explore the mechanisms of intra-oral starch incorporation and retention in dental calculus. The dental calculus model presented in this study is uniquely suited to explore these questions and may improve interpretations of dietary practices in past populations.

Acknowledgements

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