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

Bjørn Peare Bartholdy [1*], Amanda G. Henry [1]

[1] Archaeological Sciences, Leiden University, Leiden, Netherlands

Correspondence*:
Bjørn Peare Bartholdy
b.p.bartholdy@arch.leidenuniv.nl

2 ABSTRACT

3 Dental calculus has proven to contain a wealth of information on the dietary habits of past
4 populations. These insights have, to a large extent, been obtained by the extraction and
5 identification of starch granules contained within the mineralised dental plaque from a wide range of
6 regions and time periods. The scope of previous studies have been limited to microfossil extraction
7 and identification to reconstruct dietary preferences from the archaeological record, and few studies
8 have attempted to address the biases of starch retention in dental calculus. Those that have
9 considered this problem have been limited to in vivo studies on modern humans and non-human
10 primates. Here, we present a multispecies oral biofilm model, which allows experimental research
11 on starch incorporation and retention to be conducted on in vitro dental calculus in a controlled
12 laboratory setting. The biofilms were exposed to treatment solutions with known quantities of
13 dietary starches (wheat and potato) during the 25-day growth period. After this, the starch granules
14 were extracted from the mature biofilm (by dissolution in EDTA), and counted. We show that the
15 granule counts extracted from the model dental calculus represented a low proportion (ranging from
16 0.06% to 0.16%) of the total number of granules exposed to the biofilms throughout the experiment.
17 Additionally, we found that the ratios of granule sizes from the extracted starch granules differed
18 from the original treatment solutions, with large granules (>20 μm) consistently being under-
19 represented. We also found a correlation between the absolute granule counts and dry-weight of
20 the biofilm (r = 0.659, 90%CI[0.463, 0.794]), as well as between the concentration (count per mg)
21 of granules and dry-weight (r = 0.3, 90%CI[0.0618, 0.506]).
22 Our results reinforce previous in vivo studies suggesting that dental calculus presents a very small,
23 and partly biased picture of the original dietary intake of starches, with an over-representation of
24 plants producing granules smaller than 20 μm in size. The experimental model presented here is
25 well-suited to address the need for further validation of methods and biases associated with dietary
26 research on dental calculus.

27 Keywords: oral biofilm model, starch, alpha-amylase, dental calculus, paleodiet reconstruction

1 INTRODUCTION

28 Dental calculus has proven to contain a wealth of dietary information in the form of plant
29 microfossils (Henry and Piperno, 2008; Hardy et al., 2009), proteins (Hendy et al., 2018; Warinner
30 et al., 2014a), and other organic residues (Buckley et al., 2014). This dietary information can be

31 preserved within the mineralised dental plaque over many millennia, providing a unique window
32 into the food-related behaviours of past populations (Henry and Piperno, 2008; Jovanović et al.,
33 2021; Tao et al., 2020) and extinct species (Henry et al., 2014; Hardy et al., 2012).

34 Until recently, only a few studies directly investigated the presence of plant microremains in
35 the dental calculus of archaeological remains. The ability to extract phytoliths from the dental
36 calculus of archaeological fauna to investigate diet was first noted by Armitage (1975), and later
37 by Middleton and Rovner (1994), and Fox and colleagues (1996). Starches and phytoliths were
38 extracted from human dental calculus by Cummings and Magennis (1997).
39 In more recent years, the study of dental calculus has increased exponentially, and the wealth
40 of information contained within the mineralised matrix has largely been acknowledged. The use
41 of dental calculus spans a wide variety of archaeological research areas, such as oral microbiome
42 characterisation (including pathogens) through the analysis of DNA and proteins (Adler et al.,
43 2013; Warinner et al., 2014b), microbotanical remains (Henry and Piperno, 2008; Hardy et al.,
44 2009; Mickleburgh and Pagán-Jiménez, 2012), other organic residues and proteins from dietary
45 compounds (Buckley et al., 2014; Hendy et al., 2018), and nicotine use (Eerkens et al., 2018).
46 Especially the extraction of starch granules has become a rich source of dietary information, as
47 starch granules have proven to preserve well within dental calculus over a variety of geographical
48 and temporal ranges (Piperno and Dillehay, 2008; Henry et al., 2014; Tao et al., 2020; Jovanović
49 et al., 2021).

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

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

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

2 MATERIALS AND METHODS

2.1 Biofilm formation

83 In this study we employ a multispecies oral biofilm model following a modified protocol from
84 Sissons and colleagues (1991) and Shellis (1978). In brief, a biofilm inoculated with whole saliva
85 was grown on a substrate suspended in artificial saliva, and fed with sugar (sucrose). After several
86 days of growth, the biofilm was exposed to starch solutions. Mineralisation of the biofilm was
87 aided by exposure to a calcium phosphate solution. After 25 days of growth, the mineralised
88 biofilm was collected for further analysis. The setup comprises a polypropylene 24 deepwell PCR
89 plate (KingFisher 97003510) with a lid containing 24 pegs, which is autoclaved at 120°C, 1 bar
90 overpressure, for 20 mins. The individual pegs were the substrata on which the calculus grew. Using
91 this system allowed for easy transfer of the growing biofilm between saliva, feeding solutions, and
92 mineral solutions.

93 The artificial saliva (AS) is a modified version of the basal medium mucin (BMM) described by
94 Sissons and colleagues (1991). It contains 2.5 g/l partially purified mucin from porcine stomach
95 (Type III, Sigma M1778), 5 g/l trypticase peptone (Roth 2363.1), 10 g/l proteose peptone (Oxoid
96 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
97 mmol/l Na_2HPO_4 (Sissons et al., 1991), 6.4 mmol/l $NaHCO_3$ (Shellis, 1978), 2.5 mg/l haemin.
98 This is subsequently adjusted to pH 7 with NaOH pellets and stirring, autoclaved (15 min, 120°C,
99 1 bar overpressure), and supplemented with 5.8 μmol/l menadione, 5 mmol/l urea, and 1 mmol/l
100 arginine (Sissons et al., 1991).

101 Fresh whole saliva (WS) for inoculation was provided by a 31-year-old male donor with no history
102 of caries, who abstained from oral hygiene for 24 hours. No food was consumed two hours prior to
103 donation and no antibiotics were taken up to six months prior to donation. The saliva was filtered
104 through a sterilised (with sodium hypochlorite, 10–15% active chlorine) nylon cloth to remove
105 particulates. Substrata were inoculated with 1 ml/well of a two-fold dilution of WS in sterilised
106 20% (v/v) glycerine for four hours at 36°C, to allow attachment of the salivary pellicle and plaque-
107 forming bacteria. After initial inoculation, the substrata were transferred to a new plate containing
108 1 ml/well AS and incubated in a shaking incubator (Infors HT Ecotron) at 36°C, 30 rpm. The
109 inoculation process was repeated on days 3 and 5. AS was partially refreshed once per day and
110 fully refreshed every three days, throughout the experiment, by transferring the substrata to a
111 new plate containing stock AS. To feed the bacteria, the substrata were transferred to a new plate,
112 containing 5% (w/v) sucrose, for six minutes twice daily, except on inoculation days (days 0, 3,
113 and 5), where the samples only received one sucrose treatment after inoculation.

114 Starch treatments were initiated on day 9 to avoid starch granule counts being affected by α-
115 amylase hydrolysis from saliva inoculation days. An α-amylase (EC 3.2.1.1) activity assay was
116 conducted to confirm that no amylase was present in the model before starch treatments started.

117 Starch treatments replaced sucrose treatments, occurring twice per day for six minutes. The starch
118 treatments involved transferring the substrata to a new plate containing a 0.25% (w/v) starch from
119 potato (Roth 9441.1) solution, a 0.25% (w/v) starch from wheat (Sigma S5127) solution, and a 0.5%
120 (w/v) mixture of equal concentrations (w/v) wheat and potato. All starch treatments were created
121 in dH_2O with 5% (w/v) sucrose. Before transferring biofilm samples to the starch treatment plate,
122 the plates were agitated to keep the starches in suspension in the solutions. During treatments, the
123 rpm was increased to 60 to facilitate contact between starch granules and biofilms.

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

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

135 2.2   Amylase activity detection

136 An α-amylase (EC 3.2.1.1) activity assay was conducted on artificial saliva samples collected from
137 the plate wells on days 3, 6, 8, 9, 10, 12, and 14. Whole saliva samples were collected on days 0,
138 3, and 5 as positive controls. Collected samples were stored at 4°C until the assay was conducted
139 on day 18. All samples and standard curves were run in triplicates on two separate plates. Positive
140 control saliva samples were compared against a standard curve containing H_2O, while artificial saliva
141 samples were compared against a standard curve containing stock AS (due to the colour of artificial
142 saliva). Two photometric readings were conducted for each plate with a 540 nm filter on a Multiskan
143 FC Microplate Photometer (Thermo Scientific 51119000). The protocol is a modified version of
144 an Enzymatic Assay of α-Amylase (https://www.sigmaaldrich.com/NL/en/technical-documents/
145 protocol/protein-biology/enzyme-activity-assays/enzymatic-assay-of-a-amylase) (Bernfeld, 1955),
146 which measures the amount of maltose released from starch by α-amylase activity. Results are
147 reported in units (U) per mL enzyme, where 1 U releases 1 μmole of maltose in 6 minutes. The
148 detailed protocol can be found here: https://www.protocols.io/view/amylase-activity-bw8jphun.

149 2.3   Treatment solutions

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

156 2.4   Extraction method

157 Extraction of starches from the calculus samples was performed by dissolving the calculus in 0.5
158 M ethylenediaminetetraacetic acid (EDTA) (Tromp et al., 2017; Le Moyne and Crowther, 2021;
159 Modi et al., 2020), and vortexing for 3 days until the sample was completely dissolved. Twenty μl
160 of sample was mounted onto a slide with an 18x18 mm coverslip. When transferring the sample to

161 the slide, the sample was homogenised using the pipette to ensure that the counted transects were
162 representative of the whole slide. The count from the slide was extrapolated to the whole sample
163 (see Supplementary Material for more detail).

164 Both wheat and potato granules were divided into three size categories: small (<10 μm), medium
165 ($10 – 20$ μm), and large (>20 μm).

166 2.5   Statistical analysis

167 Statistical analysis was conducted in R version 4.1.2 (2021-11-01) (R Core Team, 2020) and the
168 following packages: tidyverse (Wickham et al., 2019), broom (Robinson et al., 2021), here (Müller,
169 2020), and patchwork (Pedersen, 2020).

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

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

3   RESULTS

180 All samples yielded sufficient biofilm growth and starch incorporation to be included in the analysis
181 (Figure 2), resulting in a total of 48 biofilm samples (two plates of 24), 45 of which were used for
182 analysis (three samples were set aside for later analysis). Most control samples contained no starch
183 granules, while some contained negligible quantities (see Supplementary Material).

184 3.1   No amylase activity detected in the model

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

191 3.2   Treatment type had minimal effect on biofilm growth

192 A one-way ANOVA suggests that the type of starch used during the biofilm growth period had a
193 minimal effect on the growth of the biofilm (expressed as total dry weight of the sample), $F(3, 43)$
194 $= 1.16$, p $= 0.335$. A summary of sample weights is available in Table 1.

195 3.3   Starch counts

196 It was not possible to differentiate between potato and wheat starches smaller than ca. 10 μm.
197 These were counted as wheat, as we assumed that the majority of the small granules were wheat.
198 We make this assumption based on the counts of small starches in the wheat-only and potato-only
199 solutions. Of the combined amount of small starches in these two solutions, 99.2% are from wheat.

200 The separate wheat and potato solutions were made with a 0.25% (w/v) starch concentration,
201 while the mixed-starch solution was made with 0.25% (w/v) of each starch, with a total

concentration of 0.50% (w/v). The mixed treatment had the highest absolute count of starch granules in solution (mean $= 2.9 \times 10^7$), while the biofilms exposed to the wheat solution preserved the greatest number of granules (mean $= 2.77 \times 10^4$). The potato treatment had the lowest absolute counts in both the solution (3.02×10^6) and in the biofilm samples (4850) (Tables 2 and 3).

3.3.1   Proportion of available starches incorporated in samples

The proportion of total starches from the solutions that were incorporated into the samples ranged from 0.06% to 0.16%, with potato granules being more readily incorporated than wheat in both the separated- and mixed-treatment samples (Table 4). 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 0.06% of the total available starches being incorporated into the sample, compared to 0.16% in the separated wheat treatment.

3.3.2   Size ratios differ between solutions and samples

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

The difference in incorporation between the size categories resulted in a change in size ratios between the original starch solutions and the extracted samples. Large potato granules ($> 20 \ \mu$m) were most affected, with a 32.3% decrease in relative abundance in the potato-only treatment, and a 26.5% decrease in mixed treatments. Medium granules increased in relative abundance across all samples, while small granules decreased in wheat treatments and increased in potato treatments (Figure 3).

3.3.3   Biofilm weight correlated positively with extracted starch counts

Pearson's r suggests a strong positive correlation between the total weight of the biofilms and the total starch count (standardised by z-score) extracted from the samples across treatments, r = 0.659, 90%CI[0.463, 0.794], p < 0.001 (Figure 4A).

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 4B).

4   DISCUSSION

Here, we have provided a method for exploring the incorporation of dietary starches into the mineral matrix of a dental calculus biofilm model. Our results show that a very low proportion of the starches exposed to the biofilm during growth are retained in the mineral matrix, and that the size of the starch granules may affect the likelihood of incorporation. The proportions of starch granules (of all sizes) present in the extracted samples were similar across all treatments (0.06% to 0.16%), despite large differences in absolute granule counts between wheat (mean = 25,404,000) and potato (mean = 3,016,000) solutions.

The absolute counts, however, differed more visibly between treatments and was proportional with the total count of granules in the treatment solutions. Wheat and mixed solutions had the highest absolute mean count of starch granules, and also had the highest absolute mean count of starch granules extracted from the dental calculus (Tables 2 and 3). This suggests that the starches that are

244 more frequently consumed will be present in higher quantities in the dental calculus, at least prior
245 to inhumation and degradation in the burial environment. Despite the low proportion of granules
246 recovered from the model calculus (0.06% to 0.16%), the absolute counts were still substantially
247 greater than counts recovered from archaeological remains (Tromp and Dudgeon, 2015; Tromp et
248 al., 2017; Wesolowski et al., 2010), which could in part be due to the lack of oral amylase activity
249 in our model. Previous research conducted on dental calculus from contemporary humans and non-
250 human primates suggest a high level of stochasticity involved in the retention of starch granules in
251 dental calculus, and that starch granules extracted from dental calculus are underrepresented with
252 regard to actual starch intake, which is consistent with our findings (illustrated by high standard
253 deviations and low proportional incorporation). Leonard and colleagues (2015) found individual
254 calculus samples to be a poor predictor of diet in a population, as many of the consumed plants
255 were missing from some individual samples, but were present in others.
256 Power and colleagues (2015) presented similar findings in non-human primates, where phytoliths
257 were more representative of individual diets than starch granules. The size bias is also consistent
258 with the findings by Power and colleagues (2015), who found that plants producing starches 10–20
259 μm in size were over-represented; however, the representation of granules larger than 20 μm in their
260 study is unclear.

261 We have also shown that the size of the starch granules influences the likelihood of incorporation
262 into the calculus. Starch granules larger than 20 μm in maximum length were underrepresented
263 in the calculus samples compared to the original starch solutions, an effect that was consistent
264 across all three treatments. Medium granules (10–20 μm) were often over-represented (Table 4, and
265 Figure 3). Large potato granules were most affected, potentially because of the greater size-range.
266 They can reach up to 100 μm in maximum length, whereas wheat granules generally only reach up
267 to 35 μm (Gismondi et al., 2019; Haslam, 2004; Seidemann, 1966, 174–176). Granule morphology
268 may also play a role. Large wheat granules are lenticular and have a larger surface area compared
269 to volume, whereas large potato granules are ovoid and have a larger volume compared to surface
270 area (van de Velde et al., 2002; Jane et al., 1994; Reichert, 1913, 364–365; Seidemann, 1966, 174–
271 176). Another potentially important factor from our results is the size of the calculus deposit. We
272 found a strong positive correlation between biofilm size and retained starch granules (Figure 4A);
273 a result that contradicts findings from archaeological contexts (Wesolowski et al., 2010; Dudgeon
274 and Tromp, 2014). When the concentration of starch granules per mg calculus is considered, the
275 correlation is weaker, but still present (Figure 4B). While the larger deposits contain a higher
276 absolute count, our findings also suggest that they contain a slightly higher concentration of starches.
277 This may also explain the lower mean retention of starch granules in mixed treatments compared to
278 wheat treatments. Wheat treatment samples (mean = 5.53 mg) were on average larger than mixed
279 treatment samples (mean = 4.28 mg) (Table 1); and while mixed treatment solutions contained
280 the highest mean overall granule counts, wheat treatment samples had the highest mean starch
281 retention. Further research is needed to determine why this differs from previous archaeological
282 findings.

283 The mechanism by which starch granules are incorporated into plaque and calculus remains
284 largely unknown, and few studies have directly investigated potential mechanisms. We know that
285 a proportion of the starch granules entering the mouth can become trapped in the plaque/calculus,
286 and can be recovered from archaeological samples of considerable age (Henry et al., 2014; Buckley
287 et al., 2014; Wu et al., 2021). Studies have also shown that not all starch granules come from a
288 dietary source. Other pathways include cross-contamination from plant interactions in soil, such as

289 palm phytoliths adhering to the skin of sweet potatoes (Tromp and Dudgeon, 2015), or accidental
290 ingestion not related to food consumption (Radini et al., 2017; Radini et al., 2019).
291 When starch granules enter the mouth, whether through ingestion of food or accidental intake, they
292 immediately encounter multiple obstacles. It is likely that the bulk of starch granules are swallowed
293 along with the food, and are only briefly present in the oral cavity. Other granules that are broken off
294 during mastication may be retained in the dentition through attachment to tooth surfaces (including
295 plaque and dental calculus) and mucous membranes (Kashket et al., 1991; Dodds and Edgar, 1988).
296 Bacteria also have the ability to adhere to starch granules (Topping et al., 2003), which would allow
297 starches to attach to bacterial communities within the biofilm. These granules are then susceptible
298 to mechanical removal by the tongue, salivary clearance, and hydrolysis by α-amylase (Kashket
299 et al., 1996). The susceptibility of granules to hydrolysis depends on the crystallinity and size of
300 the starch granule, as well as the mode of processing. Smaller and pre-processed (e.g., cooked)
301 starch granules are more susceptible to enzymatic degradation, while dehydrated starches will have
302 a reduced susceptibility (Lingstrom et al., 1994; Franco et al., 1992; Haslam, 2004; Björck et al.,
303 1984; Henry et al., 2009). Cracks on the surface of the dental calculus, as well as unmineralised
304 islands and channels may also be able to contain starch granules (Power et al., 2014; Tan et al.,
305 2004a; Charlier et al., 2010). Starch granules that are trapped in these pockets are (at least to
306 some extent) protected from aforementioned clearance mechanisms, especially once a new layer
307 of plaque has covered the surface of the plaque/calculus. The size bias against large granules
308 (>20 μm) from both wheat and potato (Table 4) may give further credence to this incorporation
309 pathway, as the smaller starch granules have an advantage over larger granules, and can be stored
310 in larger quantities. This was also suggested by Power and colleagues (2014), who observed clusters
311 of starches within dental calculus, rather than an even distribution across the surface of the dental
312 calculus. Granules trapped in plaque/calculus may still be susceptible to hydrolysis, as α-amylase
313 has the ability to bind to both tooth enamel and bacteria within a biofilm and retain a portion of its
314 hydrolytic activity (Scannapieco et al., 1993; Nikitkova et al., 2013; Tan et al., 2004b, 2004a). After
315 the death of an individual, starches within dental calculus are susceptible to further degradation by
316 post-depositional processes, depending on burial environment (pH, temperature, moisture content,
317 microorganisms) (Henry et al., 2009; García-Granero, 2020; Haslam, 2004; Franco et al., 1992).
318 Future study should explore how burial affects the recovery of starch from the biofilm model.

319   The absence of α-amylase in the model is a limitation of this study, as the total granule counts
320 were not subject to hydrolysis. This would likely have reduced and affected the size ratios, as smaller
321 starches may be more susceptible to hydrolysis (Franco et al., 1992; Haslam, 2004). The absence
322 may also affect biofilm growth due to the lack of amylase-bacterium interactions (Nikitkova et al.,
323 2013). Conversely, the model may benefit from the absence of α-amylase, because it can allow us
324 to directly explore its effect on starch counts in future experiments, where α-amylase can be added
325 to the model in concentrations similar to those found in the oral cavity (Scannapieco et al., 1993).
326 We are able to show how absolute counts in the treatments cause a difference in incorporation.
327 However, this was merely a side-effect of the difference in the number of granules in potato and
328 wheat solutions of the same concentration (w/v). Further research should test multiple differing
329 concentrations of the same starch type. The use of EDTA may also have affected counts. While
330 previous studies have shown negligible morphological changes caused by exposure to EDTA (Tromp
331 et al., 2017; Le Moyne and Crowther, 2021; Modi et al., 2020), these studies have not considered
332 changes to separate size categories within starch types, and whether shifts in size ratios occur due
333 to exposure to the pre-treatment chemicals. The total number of granules on a slide often exceeded

334 a number that was feasible to count in a reasonable time period, so we calculated the total counts
335 by extrapolating from three slide transects. Thus, we reasonably assume that the three transects
336 are a good representation of the entire slide, and that the distribution of all granules on the slide
337 is relatively homogeneous.
338 Finally, we only used native starches in the experimental procedure and the results will likely differ
339 for processed starches (García-Granero, 2020). Based on the comparatively low counts obtained
340 by Leonard and colleagues (2015, Supplement 2), processing and amylase may have a substantial
341 effect on starch granule retention in the oral cavity.

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

5   CONCLUSIONS

356 This preliminary study shows that a very small proportion of the input starch granules are retained
357 in a dental calculus model. This and previous studies have shown that calculus has a low capacity
358 for retention of starch granules, an effect that is compounded by diagenetic effects in archaeological
359 remains, resulting in low overall counts of extracted granules. The proportion of starches consumed
360 will in many cases be reflected in the quantity of starches extracted from the dental calculus—i.e.,
361 the more starch granules entering the oral cavity, the more will be recovered from extraction—at
362 least in modern calculus samples unaffected by diagenesis and hydrolysis. Whether or not this also
363 applies to archaeological samples remains to be tested. Additionally, we have shown that the size of
364 granules will influence the likelihood of incorporation, as large (>20 μm) starches have a decreased
365 incorporation rate, medium (10–20 μm) starches an increased rate, and small (<10 μm) granules
366 remained somewhat constant. The size of calculus deposit also seems to influence the capacity of
367 granule incorporation; as the size of the deposit increases, so does the absolute count of incorporated
368 granules.
369 While we have shown multiple factors that influence the likelihood of incorporation, the process still
370 appears to be somewhat stochastic. Further research is needed to make sense of the contributing
371 factors, and to explore the mechanisms of intra-oral starch incorporation and retention in
372 dental calculus. The oral biofilm model described in this study provides a method to explore
373 the incorporation and extraction of dietary compounds from dental calculus in a controlled
374 laboratory setting, and unearth the potential biases associated with dietary research conducted
375 on archaeological dental calculus.

OPEN SCIENCE STATEMENT

All scripts and data used in the analysis are available on OSF (https://osf.io/uc5qy/) and Github (https://github.com/bbartholdy/byoc-starch), following the format provided by the rrtools package (Marwick, 2019). More detailed protocols are available on OSF (https://osf.io/akevs/) and protocols.io (https://www.protocols.io/workspaces/byoc). Additional tables and figures are available in the Supplementary Material (https://osf.io/ucxsv/).

AUTHOR CONTRIBUTIONS

AGH acquired the funding for the analysis. BPB and AGH conceptualised and designed the study. BPB conducted the experiments and analysed the data. BPB wrote the manuscript. AGH reviewed and edited the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1. Summary statistics for biofilm dry-weights (in mg) by treatment.

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

Table 2. Mean starch counts from solutions, including the proportional makeup of the different sizes of granules.

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

Table 3. Mean starch counts extracted from samples with standard deviation (SD), including the proportion of granule sizes of the total count.

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

Table 4. The mean percentage of starches from the solutions that were incorporated into the samples.

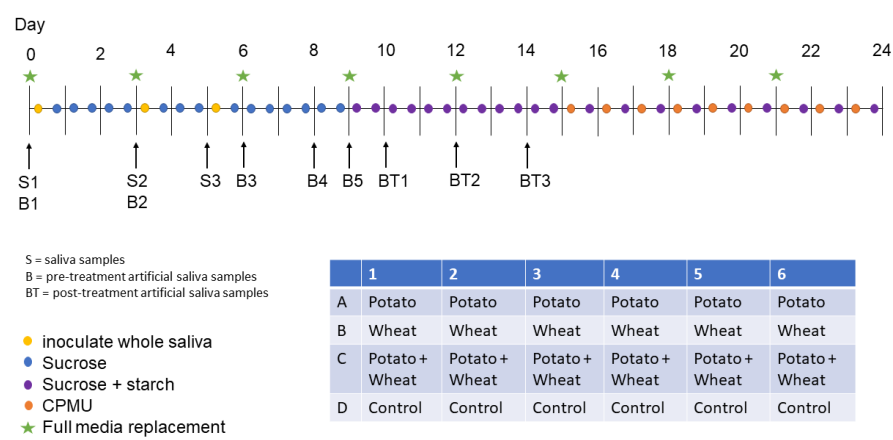| Treatment	Starch	Small	Medium	Large	Total
mix	potato		0.19%	0.05%	0.12%
mix	wheat	0.05%	0.10%	0.08%	0.06%
mix	both	0.05%	0.11%	0.07%	0.07%
potato	potato	0.28%	0.27%	0.06%	0.16%
wheat	wheat	0.09%	0.19%	0.07%	0.12%

TABLES

FIGURES

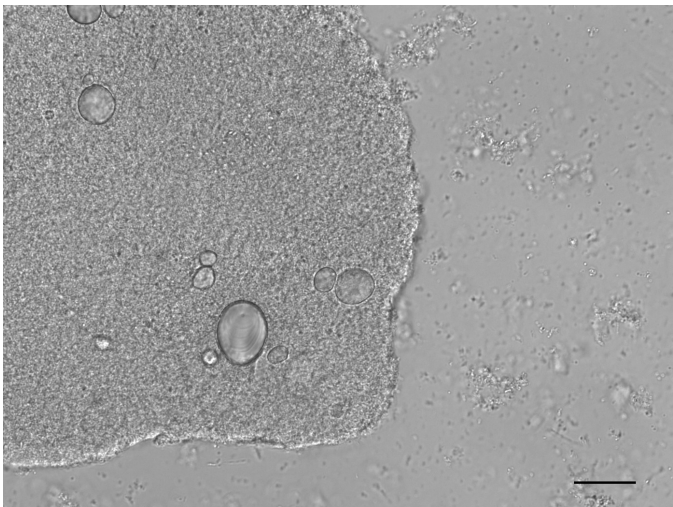Figure 1. Overview of experiment protocol including the plate setup.

Figure 2. Microscope image of a biofilm sample exposed to the potato starch solution. Potato granules can be seen within a bacterial community. Scale bar = 20 μm.
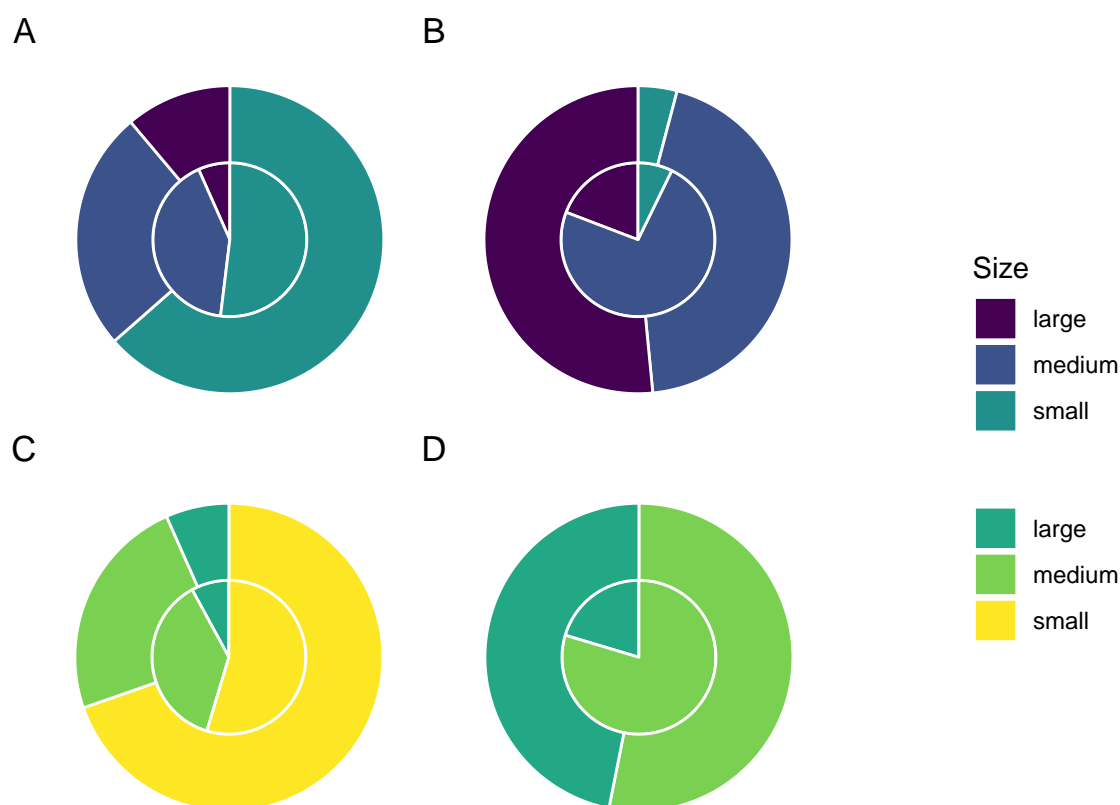
Figure 3. Proportion of sizes of starch granules from solutions (outer ring) and treatment samples (inner ring) in separated wheat (A) and potato (B) treatments, and mixed wheat (C) and potato (D) treatments.
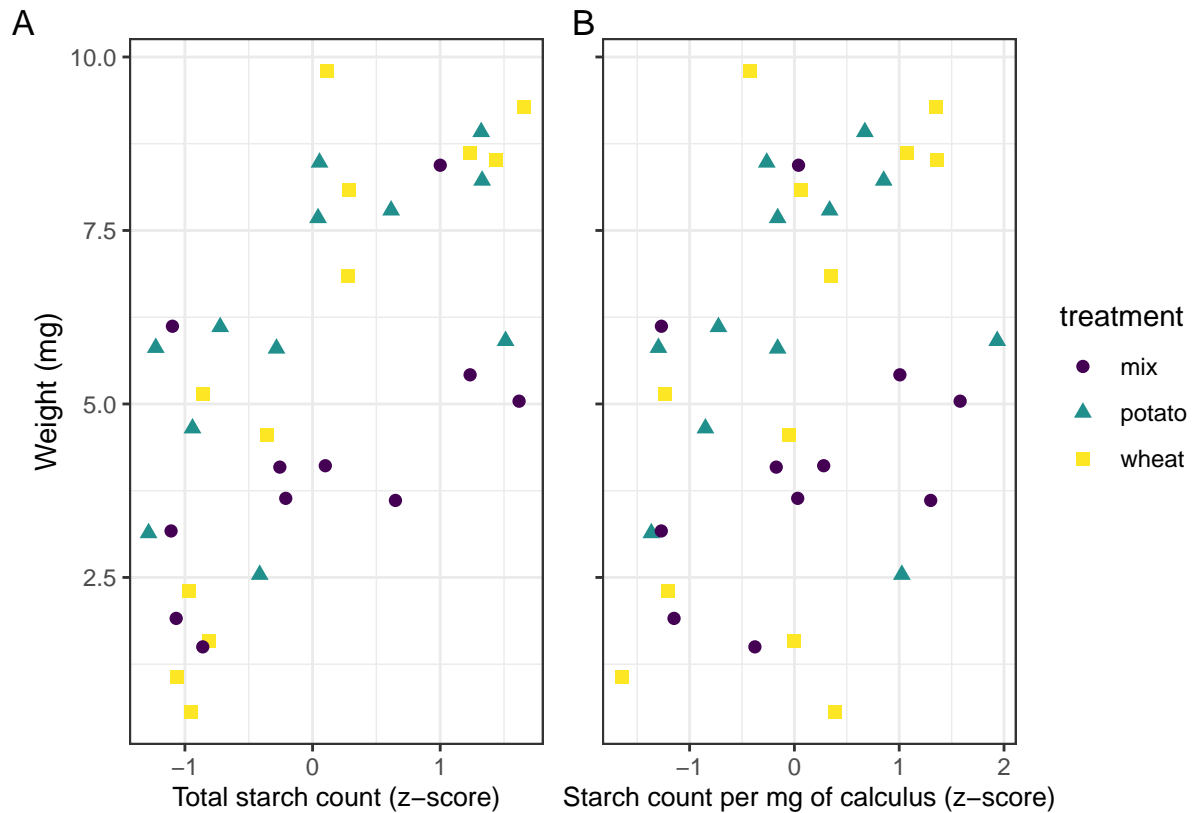
Figure 4. Scatter plots of (A) sample weight in mg and standardised starch count by z-score for separated treatments, and (B) sample weight in mg and standardised count of starch grains per mg calculus.