

1 Archaeological implications of the digestion of starches by soil bacteria: interaction among
2 starches leads to differential preservation

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7

8 **Abstract:**

9 Soil bacteria damage and destroy starch granules in archaeological contexts, but most studies of
10 this kind of damage report on pairings of a single bacterial species with starches from a single
11 plant species. Here we report the results of experiments in which starch granules from multiple
12 plants were digested by a community of soil bacteria. The damage patterns of this bacterial
13 community generally match those for single bacterial strains, and vary among plant species.
14 However, when the bacteria are exposed to a mixture of starches from different taxa, certain
15 plants are digested in favor of others. This variation in digestion could lead to a bias in the
16 starches represented in the archaeological record. The types of damage observed in this
17 experiment are further compared against that observed on archaeological starches recovered
18 from dental calculus and stone tools.

19

20 **1. Introduction**

21 Starch granules are increasingly used as markers of past human diet and behaviors. They have
22 been recovered from dental calculus, sediments, and stone and ceramic artifacts (e.g., Balme and
23 Beck, 2002; Crowther, 2005; Henry et al., 2011; Power et al., 2015). However, questions still
24 remain about how starches enter and are preserved within the archaeological record (e.g., Barton,
25 2009; Barton and Matthews, 2006; Collins and Copeland, 2011; Henry, 2015; Langejans, 2010).
26 Starches are vulnerable once exposed to soils, and are known to be decomposed by α -amylases
27 (Fuwa et al., 1977; Leach and Schoch, 1961) commonly produced by soil bacteria, such as those
28 found in the genus *Bacillus* (Sundarram and Murthy, 2014). As Haslam (2004) highlighted in his
29 review of starch decomposition in soils, the mechanisms by which starches survive this process
30 are unknown. He suggested that few starch granules out of the billions that are introduced into
31 the soil survive just by coincidence. Haslam also speculated that the formation of aggregates

32 within soils or the sequestration of starches within fissures in artifacts might protect them from
33 bacterial damage. However, more than 10 years since this seminal review there has been little
34 work by the archaeological community to understand how and why starches are preserved in
35 archaeological contexts. We need to explore in which circumstances starches may preserve, and
36 also whether taphonomic issues, such as bacterial preferences, might bias the starch record
37 against certain plant taxa. It has been long understood that different amylases are more effective
38 than others at digesting starches (e.g., Leach and Schoch, 1961; Sheets, 2016), and that the
39 starches from certain plant species or landraces are more resistance to amylolysis than others
40 (Haslam, 2004 and citations therein; Leach and Schoch, 1961; Sheets, 2016). These differences
41 have to do with the biological function and ecological niche of the amylase-producing bacteria
42 (Sheets, 2016), and the physical (e.g., size and shape) and biochemical (e.g., percentage of
43 amylose, see Cone and Wolters, 1990) features of the starches (Cone and Wolters, 1990;
44 MacGregor and Ballance, 1980; Singh et al., 2003). However, all of these studies present the
45 interactions between single starches and single amylases, and do not explore starch degradation
46 under more realistic conditions where multiple bacterial species and starches from multiple plant
47 taxa might interact. There is reason to believe that the combined effect of the soil microbiome
48 and the preference of amylases for starch from certain taxa might lead to unusual patterns of
49 starch preservation.

50 In this study, we have assessed degradation of starches from four taxa (wheat, maize, potato
51 and bean) both individually and mixed together, by a mixture of unknown soil bacteria derived
52 from local ‘living’ soils. The results from this study confirm the patterns noted previously, that
53 certain starches are more resistant to amylolysis than others, but additionally our results indicate
54 that the mixture of different starches can provide weak additive effects of degradation to some
55 starches. In light of these results, researchers must be aware of the differential preservation of
56 starches from different taxa when attempting to interpret the archaeological starch record.

57

58 **2. Materials and methods**

59 We first produced a suspension of active soil bacteria, into which we mixed starches from
60 different plant sources – wheat, potato, maize and mung bean. These starches have diverse
61 morphological and biochemical features (BeMiller and Whistler, 2009; Buléon et al., 1998;
62 Douzals et al., 1996), and represent taxa which are important nutritionally both today and in the

63 past (e.g., Babot, 2011; Piperno et al., 2004). The starch : bacteria mixtures were allowed to
64 incubate for several days, with samples extracted every 24 hours for visual microscopic
65 inspection, in order to determine the amount of damage and hydrolysis due to amylase activity.
66 The test runs were repeated five times, running for slightly different lengths each time. We then
67 re-examined our large data base of starch granules recovered from archaeological and
68 experimental contexts to see if we could identify evidence of bacterial enzymatic damage, and to
69 use the information from this study to interpret our results.

70 **2.1 Extraction of bacteria from soil.**

71 Soil was collected in Methau (Saxony, Germany) from an agriculturally-maintained hay
72 meadow (5-30 cm deep) and stored at 4°C. Before the bacterial extraction started, the soil was
73 allowed to acclimatize to room temperature overnight. It was then sieved through a 1000 µm
74 sieve (Retsch, Haan, Germany) to remove large particles. Four grams of the sieved soil were
75 milled in crushed ice with a tube mill (Tube Mill control, IKA, Staufen, Germany) using single
76 use grinding beakers (MT 40.100, IKA, Staufen, Germany) at 25000 rpm in short bursts for two
77 minutes. After this, soil suspension was transferred to sterile 50 ml tubes (Roth, Karlsruhe,
78 Germany) and centrifuged in a Heraeus centrifuge (Megafuge 16, VWR, Darmstadt, Germany) at
79 1000 rpm for 10 min to remove the big particles. The supernatant was transferred to new 50 ml
80 tube and centrifuged again at 3000 rpm for 10 min. Then, the supernatant was discarded and the
81 pellet suspended in 10 ml ddH₂O. This soil bacteria suspension was used for all further
82 experiments and stored at 4 °C when not in use.

83 **2.2 Preparation of bacteria culture**

84 Prior to each test run, the prepared soil suspension was well mixed, and 100 µl was
85 transferred to a bacteria cultivation tube (CASO-Bouillon 146432, 9 ml Mibius, Düsseldorf,
86 Deutschland) and incubated at 37 °C in an incubator (Sedona, Berlin, Germany) for about 48 h.
87 The temperature is on the high end of the preferred range (20-40 °C) for the mesophilic bacteria
88 in our soils, but this temperature at least somewhat inhibited fungal growth (Pietikäinen et al.,
89 2005). Bacterial growth was checked using a light microscope (Axio Scope, Zeiss, Göttingen,
90 Germany). After about two days, many different bacteria were present and fungal hyphae were
91 observed at the bottom of the cultivation tube.

92 **2.3 Preparation of starch : bacteria suspensions**

93 We prepared 1 % (w/v) starch suspensions using four different starch sources. Three were
94 commercially prepared: wheat starch (Weizella, Kröner Weizenstärkefabrik, Ibbenbüren,
95 Germany), potato starch (Kartoffelmehl, RUF, Lebensmittelwerke, Quakenbrück, Germany), and
96 maize starch (Feine Speisestärke, RUF, Quakenbrück, Germany). The fourth, mung bean starch,
97 was prepared from whole mung beans (purchased in 2010 at Whole Foods in Washington DC) by
98 crushing with a mortar and pestle and sieving through a 150 µm sieve (Retsch, Haan, Germany).
99 We also prepared a mixed suspension containing all four starches with a final concentration of 1
100 % (w/v) (25 mg for each taxa). The starch powder was weighed using a microbalance (Analysen-
101 und Präzisionswaage APX-200, Kern und Sohn GmbH, Balingen, Germany).

102 The different sources of our starches was some cause for concern, since it was not
103 possible to determine if the starches had been damaged or treated during their separation from
104 the plant cells. In the food industry, starches are annealed or heat-moisture to improve their
105 physicochemical properties, which also may change their susceptibility to enzymes (da Rosa
106 Zavareze and Guerra Dias, 2011). However, these treatments are regularly used to create starches
107 with non-natural properties for specific applications in processed foods, such as in canned and
108 frozen foods. Starch powders intended for use as thickeners in the home kitchen (such as we
109 used) are rarely modified in this way (Mason, 2009). We contacted the companies who produced
110 our starch powders but they declined to confirm their processing methods.

111 We added 100 mg of the starch powder to a cultivation tube, along with 1 ml of the
112 bacterial suspension, and 9 ml water to reach a final concentration of 1 % starch (w/v). The
113 bacterial suspension was taken from the upper part of the original cultivation tube to avoid
114 transferring the fungal hyphae. Cultivation tubes were incubated in an incubator (Sedona, Berlin,
115 Germany) at 37 °C. After every fifth day, half of the cultivation medium was removed and
116 refilled with fresh medium (the starch remained undisturbed at the bottom of the tube).

117 Finally, we created control samples in which 100 mg of the starch powder was mixed in
118 10 ml water to create 1 % (w/v) starch suspension. The control starch samples were treated with
119 short-wave UV light (UVP UVS-26P rechargeable UV lamp, 254nm) for 2 minutes to kill
120 endogenous bacteria. The tube was then immediately capped and placed in an incubator. The
121 initial examination of the control starches showed no strong differences among the different taxa
122 in terms of number of cracked, broken or pitted granules at the start of the experiment (fig 1). We

123 chose UV light instead of ethanol because a three-day test of starches in 1% v/v ethanol showed
124 extreme damage, including cracking, breaking, and gelatinization. Furthermore, the ethanol was
125 insufficient to keep bacteria out of the samples, particularly the mung bean and mixed samples.
126 Though ethanol is often used to prevent bacterial growth in stored samples, we expect that the
127 additional stress of the incubation caused extra damage. Similar damage to starch has been
128 documented for a variety of alcohols (Hizukuri and Takeda, 1978).

129 **2.4 Visual evaluation of starch degradation by bacteria**

130 We collected subsamples of the starch : bacteria suspensions immediately after they were
131 first prepared, and then at regular intervals (between 24 h and 3 days, depending on the replicate
132 run) to observe the visible physical changes to the starches over this period. After a thorough
133 mixing, 100 µl of the mixture was transferred to a 1.5 ml microcentrifuge tube (Eppendorf,
134 Hamburg, Germany). For microscopy, 10 µl of the starch : bacteria suspensions and 10 µl 25 %
135 glycerin solution were transferred to a slide covered with a cover glass and evaluated using an
136 Axio Scope (Carl Zeiss, Göttingen, Germany) with AxioVision software (Axio Vision LE, 64 bit,
137 Carl Zeiss, Göttingen, Germany). For documentation, pictures were taken using the AxioCam
138 MRm camera (Carl Zeiss, Göttingen, Germany). Each slide was examined, and care was taken to
139 examine a random number of fields of view along an entire transect that included the center and
140 margins of the slide. For the single-starch suspensions, we counted a total of 200 starches
141 categorizing the starch granules as native (undamaged), cracked (a crack through the starch but
142 all pieces present), broken (pieces missing), pitted (ranging from small circular surface damage,
143 to entirely dissolved in the interior), or other kinds of damage (a general category for damaged
144 starches that did not fall in any of the other categories). We could not directly assess the number
145 of starches completely degraded, but instead compared the amount of time needed to find 200
146 starches. Given that the same volume of the suspension was examined at each analysis, the
147 variation in the number of starches in this volume should reflect what was going on overall in the
148 tube. We did not explicitly time how long it took to examine each slide, however, and only have
149 the overall impression of the daily effort needed to examine the slides. In the mixed sample, we
150 counted to a total 400 starches, including only those starches which could easily be identified to
151 species. As most of the damage was apparent on larger, more diagnostic starches, this did not
152 bias our results compared to the single-starch suspensions. The experiment was repeated in five
153 independent test sets with different durations (Table 1). Three of the test sets ran only from

154 Monday through Friday. Two additional long-term tests were made with observations running for
155 more than seven days. These long-term experiments were stopped when either no starch was left,
156 or there were visually observable differences in the amount of bacteria among the five different
157 cultivation tubes.

158 The control samples were likewise sampled once per day for four days and the starches
159 counted. The samples were exposed to short wave UV light for 2 minutes after sampling before
160 being re-sealed and placed in the incubator.

161 (Table 1 goes here)

162

163 **2.5 Comparison to archaeological and experimental starch granules**

164 The enzymatically damaged starch granules produced over the course of this study displayed
165 unique morphologies that were distinct from both native granules and from damaged caused by
166 other processes such as cooking, grinding or freezing (e.g., Babot, 2003; Babot and Apella, 2003;
167 Henry et al., 2009; Messner and Schindler, 2010). In order to assess whether enzymatically
168 damaged starches could be identified in the archaeological record, or if they even survived in the
169 archaeological record, we reassessed many hundreds of starch granules recovered from various
170 archaeological contexts (e.g., Henry et al., 2014), and from experimental work involving the
171 year-long burial (Debono Spiteri et al., 2014). We looked for damage patterns, such as pitting,
172 that matched those seen in the starches from this study. We additionally examined whether, when
173 we observed enzyme-damaged starches on an archaeological or sample, the number of starch
174 types recovered was higher or lower than average on other tools from the same site. An increased
175 number of starches and starch types would suggest that pitted starches survive only in conditions
176 of overall good preservation.

177

178 **3. Results and discussion**

179 **3.1. Susceptibility of starch from different taxa to digestion by bacteria**

180 Our initial examination of starch from the four taxa, and the control samples, provided
181 the baseline against which we compared the changes due to amylase digestion. In the initial
182 control samples and in the initial samples of all five replicate tests, the wheat, potato and maize
183 starch suspensions contained more than 90 % native starches. In the control samples and on
184 average across the five replicate tests, the mung beans also had more than 90 % native starches,

185 though in two replicates we identified a greater percentage of damaged starches in the initial
186 bean flour. Mung bean starches have a large, variable mesial longitudinal cleft fissure that is
187 sometimes difficult to distinguish from cracking damage. Furthermore, we had to grind the beans
188 ourselves, making the bean flour more variable than those of the other, commercially-prepared
189 flours. Both of these factors contribute to the increased variability in the initial bean starches.

190 Given the nearly ideal conditions for bacterial growth (aqueous suspension of readily
191 available starch, and warm constant temperatures), and our lack of a completely germ-free
192 laboratory, it was impossible to keep our control samples free from bacteria, despite using UV
193 light. The mung bean and mixed samples were particularly affected by bacterial growth, though
194 pitted starches appeared in low numbers in all samples after only 24h of incubation time (figs 1
195 and 2).

196 (Figure 1 and 2 here).

197 Among our test samples, we also observed pitted granules in wheat, maize and mung
198 bean after 24h. Maize showed considerable damage with about 20 % of the granules affected,
199 while wheat and mung beans had somewhat fewer pitted granules, averaging about 15 % (fig 3 a,
200 b, c). In both wheat and mung bean, we noted that the bacterial attack occurred first on those
201 granules which were already damaged. The partially gelatinized, cracked and broken starches we
202 had observed in the initial samples either showed significant pitting damage or disappeared
203 entirely from the assemblage after the first 24 h. Over the entire course of the experiment, these
204 damaged starches represented only 0-5 % of the assemblage, with the highest values directly at
205 the beginning of the test series. This contrasted to the pattern seen in the control samples, where
206 cracked and broken starches remained a low but constant number throughout the experiment. In
207 contrast to maize, wheat and mung bean, almost all of the potato starches were still native after
208 24 h, with only a few being cracked. The proportion of cracked potato starches fluctuated
209 throughout the experiment and did not correlate with incubation time, as we also observed in the
210 control samples. The swift action of amylases on damaged starches is unsurprising, given
211 previous work demonstrating that mechanical and oxidative damage makes starch more
212 susceptible to enzymatic degradation (Haslam, 2004), and that in some cases damage from α -
213 amylases occurs after only two hours (Fuwa et al., 1977, Leach and Schoch., 1961).

214 As the experiment progressed, the proportion of pitted starch granules for all four taxa
215 increased continuously until about eight days of cultivation (fig. 3 a-d), though each species

216 showed different rates of increase. For example, after four days, more than 80 % of the maize
217 granules were damaged, while in all other species less than 50 % were damaged. Though several
218 publications have noted that some starches are more susceptible to amylases (for a review, see
219 Haslam, 2004), ours is the first study to our knowledge that shows a faster digestion of maize
220 than of wheat. This may be due to the particular bacterial amylases in our sediment samples,
221 which may have different selectivity than those studied previously, though we cannot rule out the
222 possibility that the starches were prepared using different methods that might have changed their
223 relative susceptibility to amylase. Interestingly, after ten days the percentage of damaged maize
224 granules decreased relative to native granules (fig. 3c). However, the total amount of starches
225 also clearly decreased, as noted by the amount of time needed to find 200 starches on the slide.
226 These results suggest that within maize starches, some are more resistant to bacterial attack than
227 others. Starches that were already damaged or pitted were completely degraded and consumed,
228 leaving only resistant native starches. Previous work has shown that undamaged starch granules
229 can be very resistant to enzymatic digestion (Meireles et al., 2009) and that overall digestion by
230 amylase follows an asymptotic curve after an initially quick degradation (Haslam, 2004).

231 The pattern among the other starches differed from that of maize. The proportion of
232 native wheat granules decreased continuously while the proportion of pitted granules increased.
233 After about two weeks almost all wheat granules were pitted and the total amount of granules in
234 the sample was clearly reduced, and after three weeks all granules were pitted. Mung bean
235 starches were the most affected among the samples containing only a single starch type. After 11
236 days all mung bean starch granules were pitted, and after only two weeks there were not enough
237 starches left to count to 200. In contrast, potato starches were very resistant to enzymatic attack.
238 The increase of pitted granules at the beginning was slower compared to the other taxa.
239 However, after about one week the percentage of pitted potato granules was higher than the
240 percentage of pitted mung and wheat granules. The high proportion of pitted potato starches
241 resulted mainly from surface pitting (see section 3.3 below, and figure 6 u-x, sometimes referred
242 to as “exo-corrosion”), rather than the more disruptive interior digestion (Meireles et al., 2009).
243 The proportion of granules showing interior disruption was always below 10 % until the end of
244 the experiment (data not shown) while the surface erosion increased continuously. Like in maize,
245 the percentage of native starches increased toward the end of the experiment, indicating that
246 some of the potato starches were completely resistant to degradation, while those which had been

247 pitted were completely digested. However, unlike in maize, fewer potato starches were
248 completely digested, suggesting a higher proportion of resistant starches or an overall slower
249 digestion of potato starch. While the total amount of maize starches decreased to such an extent
250 that after three weeks it was not possible to find 200 starches to count in one sample, the amount
251 of potato starches was not reduced conspicuously. The high standard variation in potato starch
252 counts between day four and five is due to the fact that the surface pitting did occur abruptly to a
253 high extent but not always at the same day within the experiment (fig. 3d).

254 (Figure 3 here)

255

256 **3.2. Enzymatic attack of starch granules in a mixed starch sample**

257 The differing levels of resistance against enzyme degradation of the starches from the
258 four investigated taxa become even more obvious in the mixed starch sample. Although potato
259 starches comprised the lowest proportion of the total starch count (due to the greater weight of
260 individual potato starches) at the start of the experiment, by about day 11 they become the
261 dominant starch, reflecting the decrease of the less-resistant starches (fig. 4). After three weeks,
262 only potato starches were detected in the mixed sample and the experiment was stopped.

263 (Figure 4 here)

264 Furthermore, we compared the behavior of the starches in the mixed vs. individual
265 samples. It appears that maize starches in the mixed sample are more affected by the bacterial
266 attack, with the proportion of pitted granules increasing far more quickly than in the individual
267 sample (fig. 5a). We observed the same trend for the wheat starches (fig. 5b). However, the
268 behavior of the wheat starches varied between the two long-term experiments. In the first run,
269 wheat starches were comparably resistant and a relevant proportion of wheat starches was left
270 until after 17 days of incubation, while in the second run all wheat starches were degraded to a
271 degree where the remaining fragments could not be confidently identified (by 5 days of
272 incubation). Previous work has shown that α -amylases from different bacterial sources have
273 different levels of activity depending on environmental conditions (Monteiro de Souza and de
274 Oliveira e Magalhães, 2010) and when exposed to starches from different taxa (Sheets, 2016).
275 Although we used the same stock suspension of soil bacteria in both experiments, we did not
276 identify the bacterial species inside each mix. It is possible that there was some incidental

277 variation in the bacterial composition in the different runs resulting in changing enzymatic
278 pattern inside the cultivation tubes.

279 In contrast to the maize and wheat starches, mung beans appeared somewhat protected by
280 the addition of other starches. In the single samples, mung beans were degraded completely after
281 11 days, but in the mixed sample they were present in a relevant proportion even after 17 days of
282 incubation (fig. 4c). These results confirm those seen elsewhere, namely that bacterial amylase
283 more readily digests starch granules from certain taxa, and, at least for the bacteria present in our
284 soil samples, maize starch is the most easily digested. Potato starches also benefited from the
285 presence of other starches, and did not appear damaged until 21 days of incubation (fig. 5d). The
286 increase of pitted granules after this time is likely due to the fact that the preferred starches had
287 already been consumed, leaving no other alternative sources for the bacteria.

288 The relative change in number of starches from different taxa in a mixed sample strongly
289 indicates that the final composition of starch granules cannot be used to predict the original
290 composition of starches. This is vital for archaeological work, where multiple starches may have
291 been present. Though our work does suggest that resistant starches may survive for longer
292 periods, we can also conclude that the relative proportion of starches from each taxon is
293 extremely likely to have changed even just few days after deposition.

294 (Figure 5 here)

295

296 **3.3. Starches degrade in a taxon-specific manner as consequence of** 297 **bacterial attack**

298 Also of archaeological relevance are the ways in which the starches from different taxa
299 are affected by bacterial enzymes. Just as with other damaging agents such as cooking and
300 processing (Henry et al., 2009), enzyme digestion causes distinct types of damage on each
301 species of starch. Wheat starches are mainly attacked from the outside to the inside, first
302 appearing as pitted or ‘chewed’. Sometimes aspects of the lamellae remained intact, resulting in
303 a striate or ringed pattern (fig. 6e and k). This seems to be a result of the enzymes preferring the
304 softer, less crystalline rings of the starches (Pérez and Bertoft, 2010; Sheets, 2016). The small
305 granules mostly show big craters from the outside to the center, resulting in a “half-moon shape”
306 that wanes until the grain is digested completely (fig. 6g). Potatoes show two different types of
307 bacterial attack. Either they are digested from the hilum, then the interior is degraded completely

308 and only the outer shell is left (fig. 6q-t)(this degradation type was described by Meireles et al.,
309 2009), or the digestion starts from the outside. In the latter case, the granules seem to be almost
310 intact with only the outer shell affected, which can be only observed when using different focus
311 layers (fig. 6u-x). Mung bean starches are usually digested beginning at the mesial longitudinal
312 cleft (fig. 7i-p). Maize granules are attacked from the outside, resulting in small round pits and
313 craters in the surface of the granules (fig. 7s-v). These differences among taxa remain even when
314 the starches are incubated together in the mixed-starch solution (fig. 8).

315 Figures 6, 7 and 8 here.

316

317 **3.4. Bacterial digestion of big and small wheat granules**

318 Wheat starch has a bimodal distribution of starch types, with spherical, ovoid or
319 polyhedral small granules ($< 8 \mu\text{m}$) without visible lamellae, and lenticular big granules ($> 8 \mu\text{m}$)
320 with clear lamellae and sometimes surface dimples or pressure marks in a golf ball-like pattern,
321 which result from the smaller granules pressing against the larger as they grow. Some authors
322 have found that smaller starches are more susceptible to digestion than larger ones (MacGregor
323 and Ballance, 1980). We therefore performed two experimental runs in which we counted the big
324 and small granules of wheat separately. The results are displayed as average values of the two
325 test sets in figure 9. At the beginning of the experiment there are more small granules than big
326 granules, and the ratio remains above one for the first four days. However, past this time the
327 amount of small granules clearly decreases and after ten days very few small granules are left.
328 The differences in values for the two experimental runs could be due to the different distribution
329 of small and big granules over the slide. While big granules mainly sit directly at the point where
330 the sample was placed, small granules distribute more evenly over the whole slide and are more
331 present at the margins. Although different areas of the slide were used for counting, the exact
332 values presented here should be considered as trend. In addition to presence and absence of the
333 two sizes, we also considered the ratio of native to pitted granules within each size class. Big
334 granules are more often pitted than small ones. After only 5 days of incubation, pitted big
335 starches outnumbered native big granules. In contrast, the visible small granules stayed native
336 until 17 days of incubation. These two ratios suggest that small granules are not as easily
337 attacked as big granules but are completely degraded once they succumb to attack. The big

338 granules are more easily damaged but survive over a longer period of time after the first attack
339 until complete degradation.

340 (Figure 9 here)

341

342 **3.5. Subjectivity in counting the different types of damage**

343 To increase the reliability of our results we tested how three different observers counted
344 the same samples. We chose an incubation time of four days to be sure that all taxa showed their
345 specific signs of bacterial attack. In figure 8 the mean values and standard deviations of three
346 individuals counting all taxa as single samples, as well as all starches in the mixed sample are
347 presented. We included only the categories native, cracked and pitted because the amount of
348 broken starches or other damage was negligible.

349 (Figure 10 here)

350 In both the counts of single taxa and in the mixed samples (fig. 10 a and 10 b) the inter-
351 observer variability is much higher for mung beans and potatoes than for wheat and corn. For
352 mung beans it was hard to differentiate among cracked, pitted and native because of the
353 variability of the mesial longitudinal cleft (see fig. 7 a-p). For potato it is easy to determine if a
354 granule is cracked but it could be difficult to separate native and pitted potato granules. As
355 mentioned before, we found two different types of pitted potatoes, one type where the interior of
356 the granule is completely degraded (fig. 6q, r) which occurred only rarely and is clearly
357 identifiable, and the other type where only the surface of the granules is affected (fig. 6u-x). This
358 type started to occur from the second to third day on. Here again there is no distinct transition
359 and the damage would not be visible if all focal layers of a starch were not carefully examined.
360 The inter-observer variability for wheat was also quite high in both the single and the mixed
361 sample. However, the differences between the categories were still always significant so that it is
362 likely that different observers would obtain the same results. This is in contrast to mung bean and
363 potato where different observers would probably reach different results.

364

365 **3.6. Enzymatic damage on archaeological and experimental starches**

366 In order to explore whether pitting damage could be observed on starch grains exposed to
367 conditions more relevant to archaeology, we re-examined data collected from two other studies.
368 For the first, we reanalyzed the starches recovered from an experiment in which starch-covered

369 stone tools were buried and therefore exposed to native soil bacteria for two years. In this
370 previous experiment, we had created retouched stone flakes and exposed them to one of seven
371 treatments: raw potato, cooked potato, raw wheat, cooked wheat, raw cattail, cooked cattail or no
372 plant (Debono Spiteri et al., 2014), with three replicates of each treatment. About 180 of these
373 flakes were buried in seven different sites around Europe and dug up after two years. Of 1488
374 starches recovered from all stone flakes, 444 displayed pitting similar to that seen in this
375 experiment (30% of the assemblage). These pitted starches were not evenly distributed
376 throughout the sample, and instead were found in large clumps on only four flakes, with some
377 clumps including more than 100 starches. All of the identified pitted starches came from
378 potatoes; none of the pitted starches could be identified as wheat or cattail, though some were
379 non-diagnostic forms. Three of the four flakes were from the same site – an agriculturally-
380 maintained meadow. These three flakes had initially been exposed to raw potato, and comprise
381 the three replicate treatments of raw potato for this site. All three also had native potato starches
382 preserved, though the ratio of native to pitted was roughly 1:2. Only six of the 18 other flakes
383 from this site showed any preservation of starches, and these were generally non-diagnostic and
384 in very low numbers (1 or 2 on each flake). The stone flakes had initially been heavily covered in
385 plant material, including presumably intact cell walls, providing an extra layer of protection to
386 the starches. This pattern was also observed in another test of buried starches (Barton, 2009).
387 Furthermore, the inherent resistance of potato starches to enzyme damage might explain why
388 some starches could show pitting but not be entirely removed from the record. It is also possible
389 that the bacterial community in the meadow were not particularly well-suited to digesting potato
390 starches. In general, we can say that pitted starches are preserved on buried stone flakes only in
391 exceptional cases. It is likely that once bacteria begin attacking starches on a flake, they make
392 quick work of destroying the starches entirely.

393 The stone flake burial study only ran for two years, and may have limited value for
394 understanding pitted starches in deep time. We reanalyzed images collected during a previously
395 published study of Neanderthal and early modern human dental calculus and stone tool samples
396 (Henry et al., 2014) in order to see whether pitted starches could be recognized in archaeological
397 samples. This study included 125 stone tools and 67 dental calculus samples from 36 individuals
398 from 19 sites ranging in age from 8 ka to at least 130 ka and possibly up to 430 ka (the age of
399 one sample is not firmly established). From these samples, we recovered 626 starch granules, of

400 which ten displayed damage that is consistent with enzyme digestion (fig. 11), comprising only
401 1.8 % of the total assemblage. Pitted starches were found on older (c. 100 ka) and younger (c. 20
402 ka) material, and from all of the main geographical regions covered in the initial study
403 (Mediterranean and northern Europe, the Near East, and central and southern Africa) (Table 2).
404 Despite this widespread preservation, the overall extremely low number of pitted starches
405 suggests that such damaged starches are unlikely to survive the long-term taphonomic processes
406 that can affect archaeological assemblages. We already observed that once bacterial action begins
407 on starches, the starches very quickly become completely digested. Even if the enzymatic action
408 pauses, the partially-digested starches are likely more susceptible to changes in temperature,
409 moisture, and pH.

410 (figure 11 here) (Table 2 here)

411 In general, it is difficult if not impossible to determine whether the pitting occurred in
412 antiquity when the starch was first used by humans, or if it is the result of post-depositional
413 bacterial action, or if it is a combination of several processes. For example, the four pitted
414 starches coming from calculus samples could have been caused by human salivary or oral
415 bacteria amylases. For the six pitted starches from stone tools, it is possible that soil bacteria
416 from the sites in which the tools were buried are the causal agents. A more remote possibility is
417 that some or all of these pitted starches might represent the processing or consumption of
418 sprouted seeds (where the starches have been damaged by endemic plant amylases). It is
419 currently not possible to identify the source of the damage-producing enzyme.

420 Our experiment clearly demonstrated that the enzyme damage to starches from particular
421 taxon varies depending on whether starches from just that taxon or a mix of starches are
422 available. This pattern further emphasizes the need to consider not only individual starches, but
423 rather to look for overall patterns within an assemblage. For each of archaeological samples with
424 pitted starches, we explored how the pattern of recovered starches compared to that on other
425 samples from the same assemblage. Assemblages were defined as samples coming from the same
426 site and level, and therefore represent a single group of people with similar diets, and also were
427 from the same sedimentary contexts and subjected to similar taphonomic processes. We
428 compared starch types rather than taxa because in many cases we were unable to identify the
429 taxonomic origin of the archaeological starches. In some cases, several types may come from one
430 plant species, and in other cases, one type may represent several taxa. As seen in table 2, we

431 found that the samples with pitted starches always had several other starches, often representing
432 different starch types. These samples were often among those with the best preservation (defined
433 as having the most starches and the most starch types) within the assemblage. However, no
434 sample had more than one pitted starch. Taken together, these patterns suggest that pitted
435 starches may be preserved only in those microenvironments which are particularly conducive to
436 general starch preservation. We cannot conclude that these pitted starches are firm evidence for
437 biasing for or against certain taxa, however. Given our experimental data from the mixed
438 samples, we might have expected a biased sample to have only one starch type represented on
439 the samples which had been affected by sedimentary bacteria (i.e., all of the other starch types
440 had been digested). In contrast, we saw that the samples with pitted bacteria were likely the ones
441 least affected by bacterial action, with the most starches and most types preserved. The low
442 numbers of starches on the samples with no pitted starches suggests that bacteria had completely
443 removed the entire starch record on those samples, leaving behind a possibly less-biased
444 assemblage on the samples with pitted starches.

445

446 **4. Conclusion**

447 Our data confirm that among the four starchy taxa we tested, potato starch was the most
448 resistant against enzymatic digestion. Importantly, we determined that the mixture of starch from
449 different taxa changes the behavior of the enzymes, resulting in differing levels of degradation.
450 In our experiments this was in particularly obvious for maize starch that was more resistant when
451 incubated alone with bacterial enzymes, and for mung bean and potato starch that were more
452 resistant in the starch mixture. Furthermore, some of the starches within each taxon were more
453 resistant against enzymatic digestion than others of the same species. These resistant starches
454 were particularly present in maize and potato. It is somewhat difficult to accurately assess
455 damage to starch, however, as indicated by the variability in our inter-observer tests.

456 All of these observations are relevant for understanding the preservation of starch granules in
457 the archaeological record, and interpreting the results of starch grain analyses. While the swift
458 digestion of starch and inter-observer variability are somewhat worrisome, our results also
459 confirm the presence of starches that are very resistant to bacterial digestion, despite our use of
460 environmental conditions that are the most conducive to bacterial damage (i.e., starches
461 dispersed in a liquid suspension, high bacterial load, very warm and constant temperatures). Such

462 growth conditions are rarely found in archaeological sites, suggesting that the rate of digestion of
463 archaeological starches would be much reduced compared to what we document here. The
464 finding of differential survival of starches in mixed samples strongly implies a need for caution
465 when interpreting the relative proportion of different starches in an archaeological sample.
466 However, the presence of starch still speaks to the presence of a particular plant type, and the
467 recovery of pitted starches in archaeological samples can attest to the action of enzymes. The
468 source of these enzymes, whether from salivary amylase, oral bacteria, soil bacteria, or
469 endogenous plant amylase, is impossible to determine. The long-term survival of pitted starches
470 is likely to occur only in exceptional cases where the bacterial activity is arrested after the initial
471 exposure, and should not be expected in most assemblages.

472

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477

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566

567 **Figure Captions:**

568 Figure 1: Degradation of starch granules in single-species control samples with no introduced
569 bacteria over a period of 4 days. Native, broken, cracked, pitted and otherwise damaged granules
570 were counted and the relative proportion of each type presented here. Only categories with a
571 relevant number of hits are displayed, and the proportion of completely destroyed granules is
572 unknown and therefore not shown. A) maize starch, b) wheat starch, c) mung bean starch, d)
573 potato starch.

574

575 Figure 2: Starch degradation mixed starch control sample over a time period of 4 days. Native,
576 broken, cracked, pitted and otherwise damaged granules were counted. Only categories with a
577 relevant number of hits are displayed. A) maize starch, b) wheat starch, c) mung bean starch , d)
578 potato starch, e) relative proportions of each starch type in the overall sample.

579

580 Figure 3: Degradation of starch granules in single-species (not mixed) samples by bacterial
581 enzymes over a period of 21 days. Native, broken, cracked, pitted and otherwise damaged

582 granules were counted and the relative proportion of each type presented here. Only categories
583 with a relevant number of hits are displayed, and the proportion of completely destroyed
584 granules is unknown and therefore not shown. A) maize starch, b) wheat starch, c) mung bean
585 starch, d) potato starch. Error bars display standard deviation of three to five replicate
586 measurements.

587

588 Figure 4: Changes through time in the relative proportions of starch granules from the four taxa
589 (mung bean, maize, wheat and potato) in a mixed sample.

590

591 Figure 5: Starch degradation by bacterial enzymes in a mixed starch sample over a time period of
592 21 days. Native, broken, cracked, pitted and otherwise damaged granules were counted. Only
593 categories with a relevant number of hits are displayed. A) maize starch, b) wheat starch, c)
594 mung bean starch, d) potato starch. Error bars display standard deviation of three to four
595 replicate measurements.

596

597 Figure 6: Native and enzyme-damaged wheat and potato starches from the single-starch
598 digestions. The left image of each pair is under brightfield and the right under cross-polarized
599 light. The scale bar in a applies to all of the wheat images and that in u applies to all of the potato
600 images. a&b: Native wheat starches. c-l: Enzyme-damaged wheat starches. m&n: native potato
601 starches; o-x: Enzyme-damaged potato starches. The damage on u-x is particularly subtle,
602 appearing only on the surface of the starch.

603

604 Figure 7: Native and enzyme-damaged mung bean and maize starches from the single-starch
605 digestions. The left image of each pair is under brightfield and the right under cross-polarized
606 light. The scale bar in a applies to all starches. a-h: native mung bean starches (note the erratic
607 and variable mesial longitudinal cleft fissure, which made identifying enzyme damage more
608 challenging). i-p: Enzyme-damaged mung bean starches. q&r: Native maize starches. s-x:
609 Enzyme-damaged maize starches.

610

611 Figure 8: Native and enzyme-damaged starches from the mixed starch digestion. The left image
612 of each pair is under brightfield and the right under cross-polarized light. Each subfigure
613 contains starches from several taxa.

614

615 Figure 9: Digestion of small and big wheat granules by bacterial enzymes. A) Ratio of small and
616 big granules over time, b) proportion of pitted and native granules for small and big wheat over
617 incubation time.

618

619 Figure 10: Mean values and standard deviations of independent counts of the same samples by
620 three different observers. a) Mean values and standard deviations obtained from single starch
621 samples, b) mean values and standard deviations after three independent counts of starches from
622 each of the four taxa in the mixed sample.

623

624 Figure 11: Starch granules from archaeological specimens from Henry et al. (2014) showing
625 damage consistent with enzyme digestion. The right side of each pair shows the starches in
626 brightfield, the left under cross-polarized light. a&b: from calculus sample Blombos 8971 (SAM-
627 AP 8971 left upper deciduous m2), c&d: from grindstone Gorham's Cave sample 6 (Gor 00 / b8 /
628 NIV / 220); e&f: from calculus sample Ishango 15 LM1 (layer NFP); g&h: from stone tool
629 Klasies River Mouth shelter 1b layer 10 tool sample 4 (bag "flake blades"); i&j: from stone tool
630 Klasies River Mouth cave 1 layer 14 tool sample 11 (bag KRM 1 [471] (14) F W. cutting 16647);
631 k&l) from calculus sample La Ferrassie I left upper M3; m&n) from stone tool Skhul sample 22
632 (#37-22-60/3199 layer b); o) from stone tool Skhul sample 4 (#37-22-60/3224 layer b1n) (no
633 cross-polarized image was taken); p) from stone tool Klasies River Mouth shelter 1b layer 10
634 tool sample 1 (bag "stone industry") (no cross polarized image was taken); q&r) from calculus
635 Spy I right lower M1 (#580c). All figure parts are at the same scale, and each of the small boxes
636 is 50 μm square.

637