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

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Abstract:

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

1. Introduction

Starch granules are increasingly used as markers of past human diet and behaviors. They have been recovered from dental calculus, sediments, and stone and ceramic artifacts (e.g., Balme and Beck, 2002; Crowther, 2005; Henry et al., 2011; Power et al., 2015). However, questions still remain about how starches enter and are preserved within the archaeological record (e.g., Barton, 2009; Barton and Matthews, 2006; Collins and Copeland, 2011; Henry, 2015; Langejans, 2010). Starches are vulnerable once exposed to soils, and are known to be decomposed by α-amylases (Fuwa et al., 1977; Leach and Schoch, 1961) commonly produced by soil bacteria, such as those found in the genus *Bacillus* (Sundarram and Murthy, 2014). As Haslam (2004) highlighted in his review of starch decomposition in soils, the mechanisms by which starches survive this process are unknown. He suggested that few starch granules out of the billions that are introduced into the soil survive just by coincidence. Haslam also speculated that the formation of aggregates within soils or the sequestration of starches within fissures in artifacts might protect them from bacterial damage. However, more than 10 years since this seminal review there has been little work by the archaeological community to understand how and why starches are preserved in archaeological contexts. We need to explore in which circumstances starches may preserve, and also whether taphonomic issues, such as bacterial preferences, might bias the starch record against certain plant taxa. It has been long understood that different amylases are more effective than others at digesting starches (e.g., Leach and Schoch, 1961; Sheets, 2016), and that the starches from certain plant species or landraces are more resistance to amylolysis than others (Haslam, 2004 and citations therein; Leach and Schoch, 1961; Sheets, 2016). These differences have to do with the biological function and ecological niche of the amylase-producing bacteria (Sheets, 2016), and the physical (e.g., size and shape) and biochemical (e.g., percentage of amylose, see Cone and Wolters, 1990) features of the starches (Cone and Wolters, 1990; MacGregor and Ballance, 1980; Singh et al.,

2003). However, all of these studies present the interactions between single starches and single amylases, and do not explore starch degradation under more realistic conditions where multiple bacterial species and starches from multiple plant taxa might interact. There is reason to believe that the combined effect of the soil microbiome and the preference of amylases for starch from certain taxa might lead to unusual patterns of starch preservation.

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

2. Materials and methods

We first produced a suspension of active soil bacteria, into which we mixed starches from different plant sources – wheat, potato, maize and mung bean. These starches have diverse morphological and biochemical features (BeMiller and Whistler, 2009; Buléon et al., 1998; Douzals et al., 1996), and represent taxa which are important nutritionally both today and in the past (e.g., Babot, 2011; Piperno et al., 2004). The starch : bacteria mixtures were allowed to incubate for several days, with samples extracted every 24 hours for visual microscopic inspection, in order to determine the amount of damage and hydrolysis due to amylase activity. The test runs were repeated five times, running for slightly different lengths each time. We then re-examined our large data base of starch granules recovered from archaeological and experimental contexts to see if we could identify evidence of bacterial enzymatic damage, and to use the information from this study to interpret our results.

2.1 Extraction of bacteria from soil.

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

2.2 Preparation of bacteria culture

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

2.3 Preparation of starch : bacteria suspensions

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

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

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

Finally, we created control samples in which 100 mg of the starch powder was mixed in 10 ml water to create 1 % (w/v) starch suspension. The control starch samples were treated with short-wave UV light (UVP UVS-26P rechargeable UV lamp, 254nm) for 2 minutes to kill endogenous bacteria. The tube was then immediately capped and placed in an incubator. The initial examination of the control starches showed no strong differences among the different taxa in terms of number of cracked, broken or pitted granules at the start of the experiment (fig 1). We chose UV light instead of ethanol because a three-day test of starches in 1% v/v ethanol showed extreme damage, including cracking, breaking, and gelatinization. Furthermore, the ethanol was insufficient to keep bacteria out of the samples, particularly the mung bean and mixed samples. Though ethanol is often used to prevent bacterial growth in stored samples, we expect that the additional stress of the incubation caused extra damage. Similar damage to starch has been documented for a variety of alcohols (Hizukuri and Takeda, 1978).

2.4 Visual evaluation of starch degradation by bacteria

We collected subsamples of the starch : bacteria suspensions immediately after they were first prepared, and then at regular intervals (between 24 h and 3 days, depending on the replicate run) to observe the visible physical changes to the starches over this period. After a thorough mixing, 100 µl of the mixture was transferred to a 1.5 ml microcentrifuge tube (Eppendorf, Hamburg, Germany). For microscopy, 10 µl of the starch : bacteria suspensions and 10 µl 25 % glycerin solution were transferred to a slide covered with a cover glass and evaluated using an Axio Scope (Carl Zeiss, Göttingen, Germany) with AxioVision software (Axio Vision LE, 64 bit, Carl Zeiss, Göttingen, Germany). For documentation, pictures were taken using the AxioCam MRm camera (Carl Zeiss, Göttingen, Germany). Each slide was examined, and care was taken to examine a random number of fields of view along an entire transect that included the center and margins of the slide. For the single-starch suspensions, we counted a total of 200 starches categorizing the

starch granules as native (undamaged), cracked (a crack through the starch but all pieces present), broken (pieces missing), pitted (ranging from small circular surface damage, to entirely dissolved in the interior), or other kinds of damage (a general category for damaged starches that did not fall in any of the other categories). We could not directly assess the number of starches completely degraded, but instead compared the amount of time needed to find 200 starches. Given that the same volume of the suspension was examined at each analysis, the variation in the number of starches in this volume should reflect what was going on overall in the tube. We did not explicitly time how long it took to examine each slide, however, and only have the overall impression of the daily effort needed to examine the slides. In the mixed sample, we counted to a total 400 starches, including only those starches which could easily be identified to species. As most of the damage was apparent on larger, more diagnostic starches, this did not bias our results compared to the single-starch suspensions. The experiment was repeated in five independent test sets with different durations (Table 1). Three of the test sets ran only from Monday through Friday. Two additional long-term tests were made with observations running for more than seven days. These long-term experiments were stopped when either no starch was left, or there were visually observable differences in the amount of bacteria among the five different cultivation tubes.

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

Table 1: The five test runs, showing the days on which the starches were visually examined.

day	0	1	2	3	4	5	6	7	8	11	14	17	21
1st run	x	x	x										
2nd run	x	x	x			x		x					
3rd run	x	x	x	x	x								
4th run	x	x	x	x	x				x	x	x	x	x
5th run	x	x	x	x	x	x	x	x	x				

All runs include individual vials of wheat, potato, maize and bean starch. Only the first test run does not include a mix of the four starch types.

2.5 Comparison to archaeological and experimental starch granules

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

3. Results and discussion

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

Our initial examination of starch from the four taxa, and the control samples, provided the baseline against which we compared the changes due to amylase digestion. In the initial control samples and in the initial samples of all five replicate tests, the wheat, potato and maize starch suspensions contained more than 90 % native starches. In the control samples and on average across the five replicate tests, the mung beans also had more than 90 % native starches, though in two replicates we identified a greater percentage of damaged starches in the initial bean flour. Mung bean starches have a large, variable mesial longitudinal cleft fissure that is sometimes difficult to distinguish from cracking damage. Furthermore, we had to grind the beans ourselves, making the bean flour more variable than those of the other, commercially-prepared flours. Both of these factors contribute to the increased variability in the initial bean starches.

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

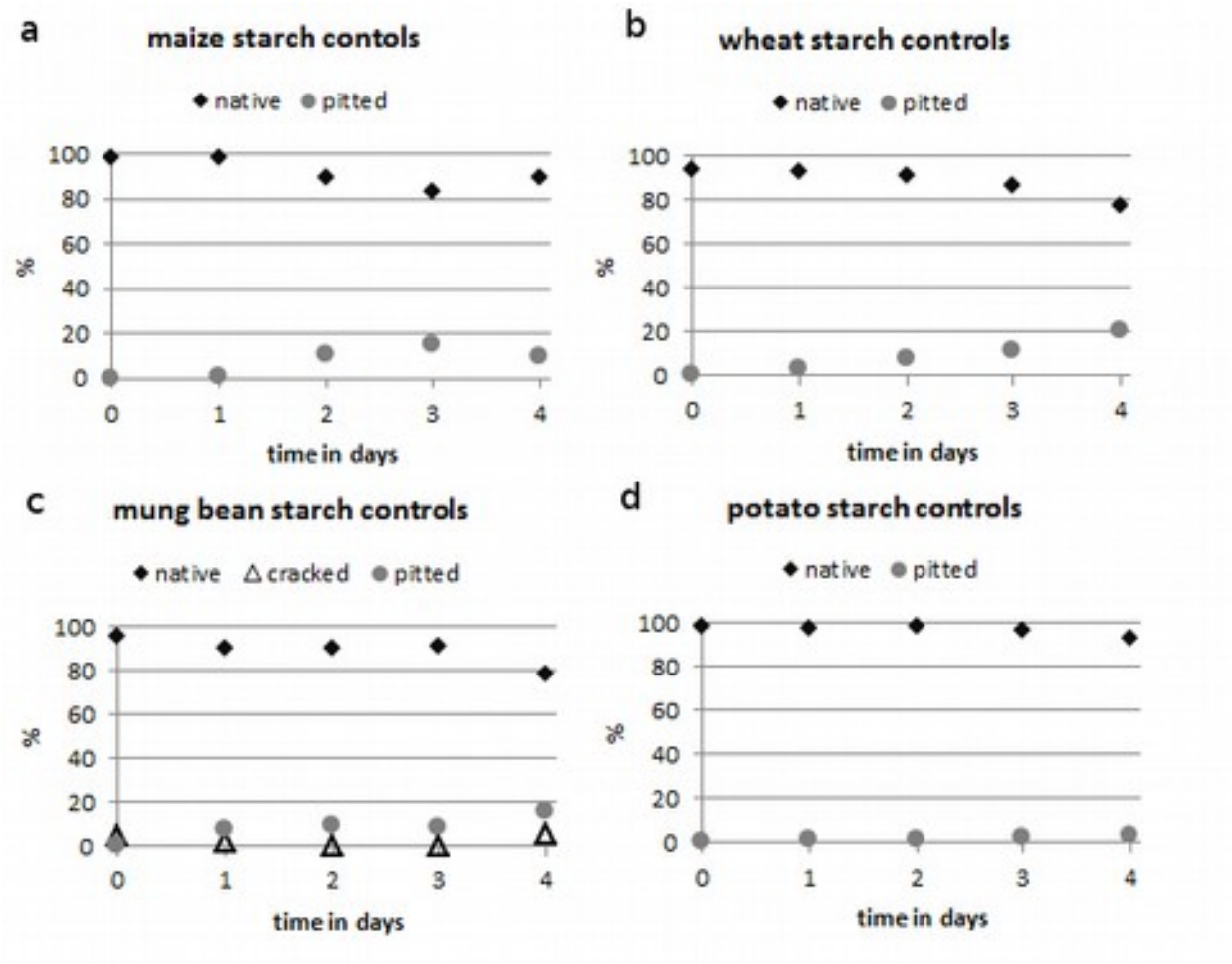

***Figure 1**: Degradation of starch granules in single-species control samples with no introduced bacteria over a period of 4 days. Native, broken, cracked, pitted and otherwise damaged granules were counted and the relative proportion of each type presented here. Only categories with a relevant number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

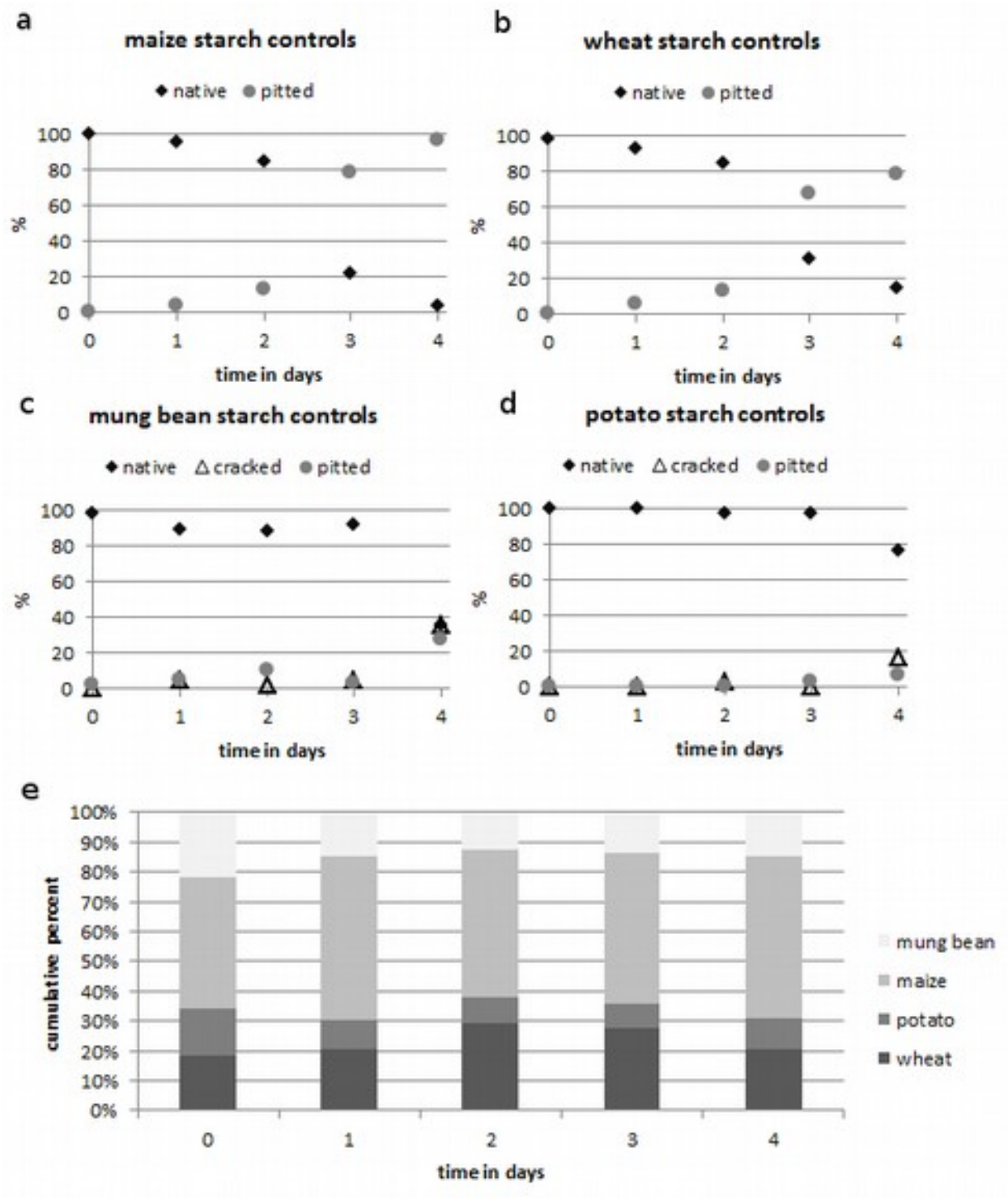

Figure 2*: Starch degradation mixed starch control sample over a time period of 4 days. Native, broken, cracked, pitted and otherwise damaged granules were counted. Only categories with a relevant number of hits are displayed. A) maize starch, b) wheat starch, c) mung bean starch , d) potato starch,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

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Among our test samples, we also observed pitted granules in wheat, maize and mung bean after 24h. Maize showed considerable damage with about 20 % of the granules affected, while wheat and mung beans had somewhat fewer pitted granules, averaging about 15 % (fig 3 a, b, c). In both wheat and mung bean, we noted that the bacterial attack occurred first on those granules which

were already damaged. The partially gelatinized, cracked and broken starches we had observed in the initial samples either showed significant pitting damage or disappeared entirely from the assemblage after the first 24 h. Over the entire course of the experiment, these damaged starches represented only 0-5 % of the assemblage, with the highest values directly at the beginning of the test series. This contrasted to the pattern seen in the control samples, where cracked and broken starches remained a low but constant number throughout the experiment. In contrast to maize, wheat and mung bean, almost all of the potato starches were still native after 24 h, with only a few being cracked. The proportion of cracked potato starches fluctuated throughout the experiment and did not correlate with incubation time, as we also observed in the control samples. The swift action of amylases on damaged starches is unsurprising, given previous work demonstrating that mechanical and oxidative damage makes starch more susceptible to enzymatic degradation (Haslam, 2004), and that in some cases damage from α-amylases occurs after only two hours (Fuwa at al., 1977, Leach and Schoch., 1961).

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

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

conspicuously. The high standard variation in potato starch counts between day four and five is due to the fact that the surface pitting did occur abruptly to a high extent but not always at the same day within the experiment (fig. 3d).

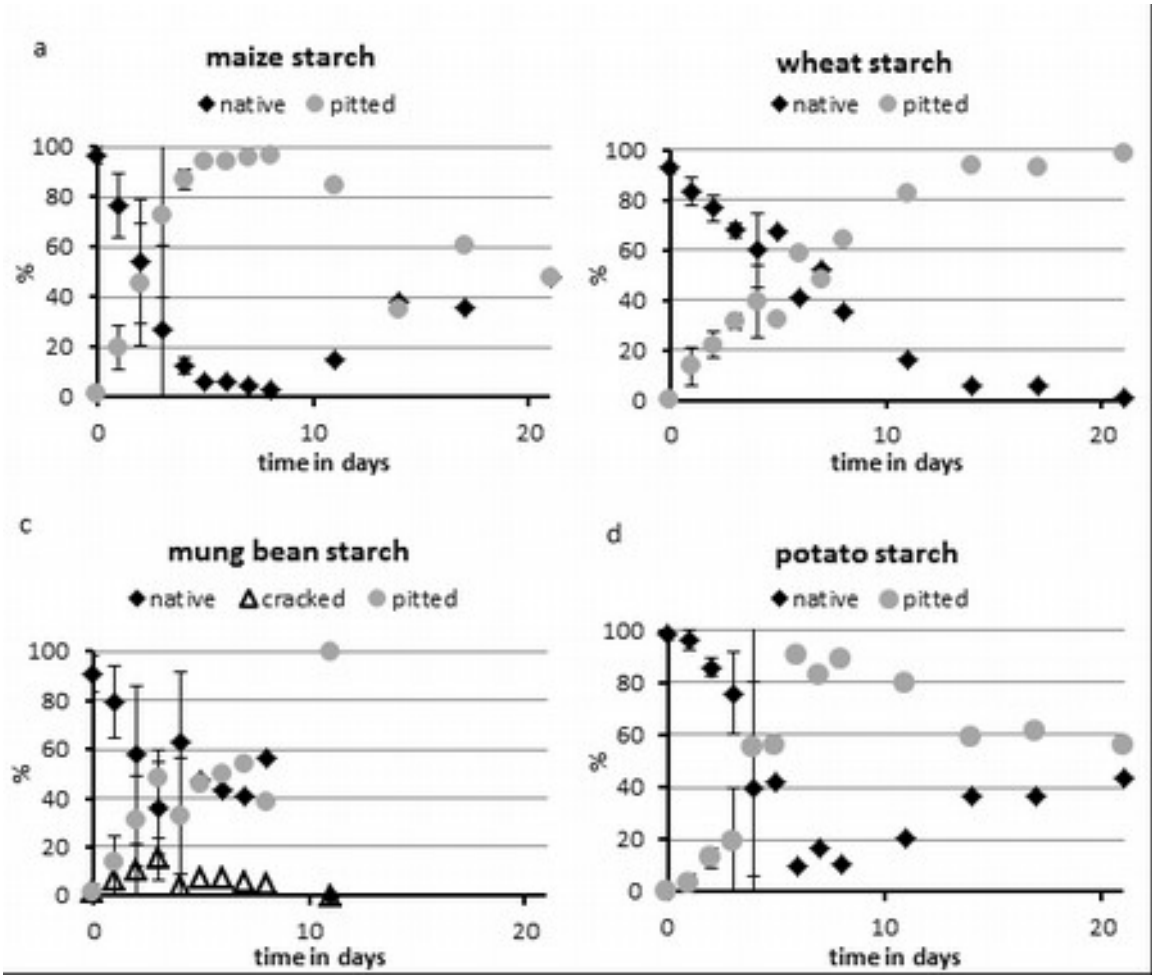

Figure 3: *Degradation of starch granules in single-species (not mixed) samples by bacterial enzymes over a period of 21 days. Native, broken, cracked, pitted and otherwise damaged granules were counted and the relative proportion of each type presented here. Only categories with a relevant number of hits are displayed, and the proportion of completely destroyed granules is unknown and therefore not shown. A) maize starch, b) wheat starch, c) mung bean starch, d) potato starch. Error bars display standard deviation of three to five replicate measurements.*

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

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

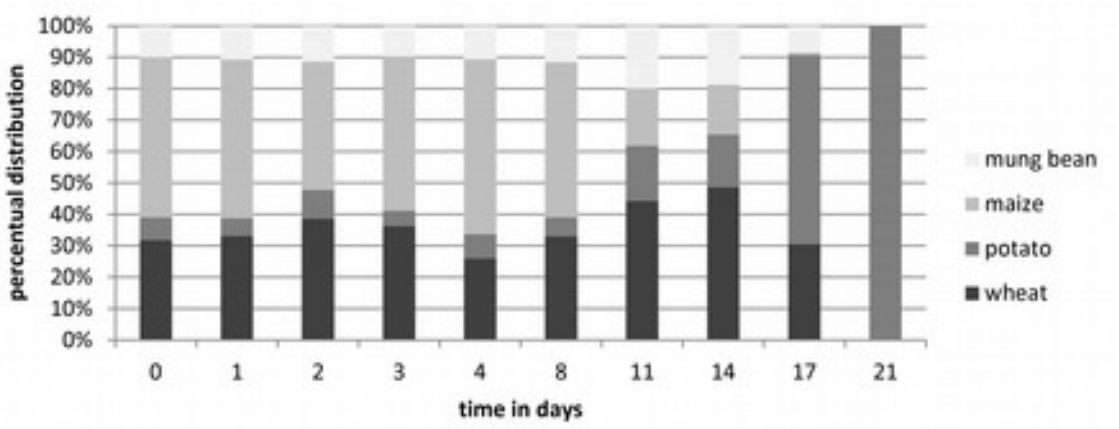

Figure 4: *Changes through time in the relative proportions of starch granules from the four taxa (mung bean, maize, wheat and potato) in a mixed sample.*

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

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

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

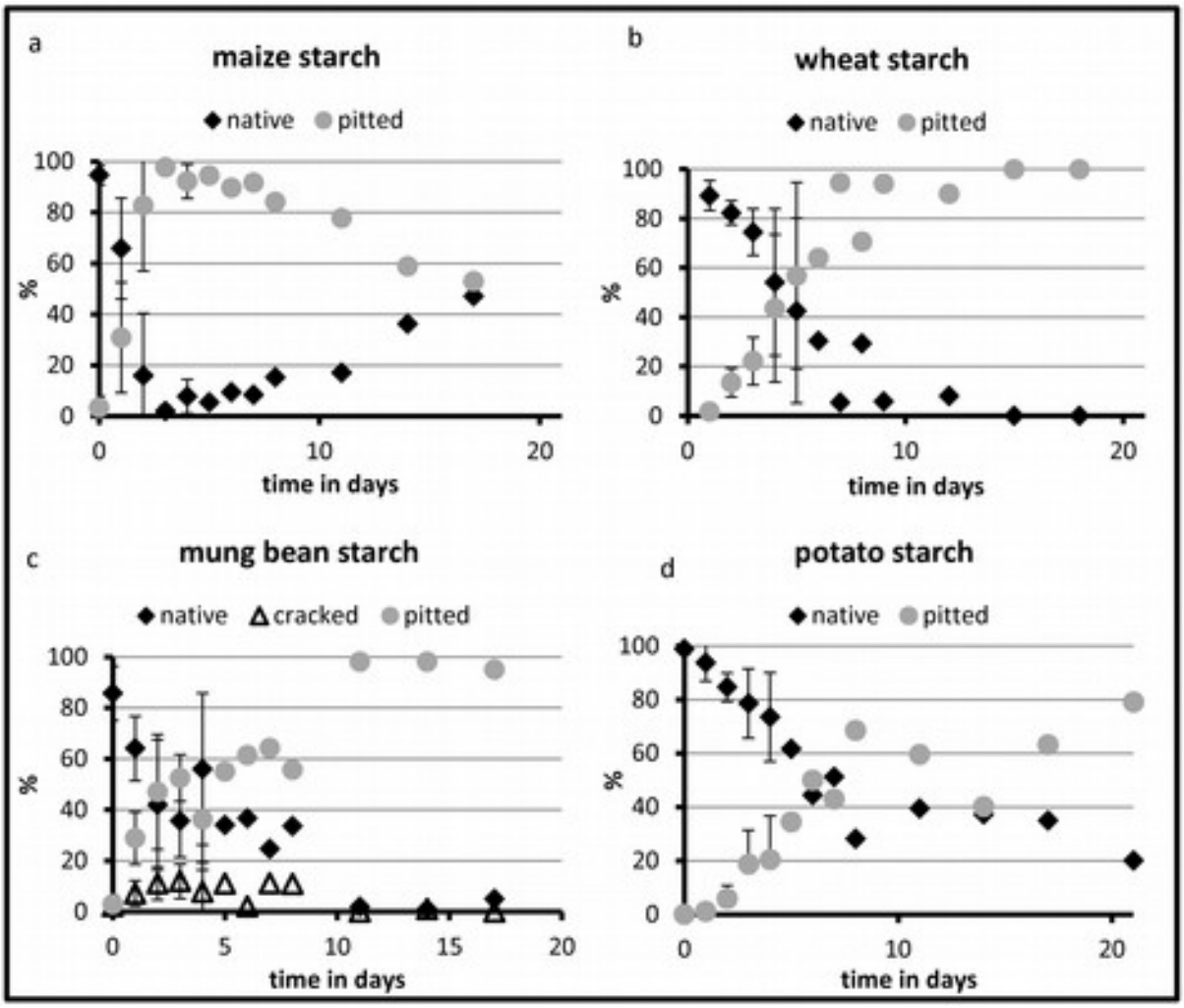

Figure 5*: Starch degradation by bacterial enzymes in a mixed starch sample over a time period of 21 days. Native, broken, cracked, pitted and otherwise damaged granules were counted. Only categories with a relevant number of hits are displayed. A) maize starch, b) wheat starch, c) mung bean starch ,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

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

Also of archaeological relevance are the ways in which the starches from different taxa are affected by bacterial enzymes. Just as with other damaging agents such as cooking and processing (Henry et al., 2009), enzyme digestion causes distinct types of damage on each species of starch. Wheat starches are mainly attacked from the outside to the inside, first appearing as pitted or ‘chewed’. Sometimes aspects of the lamellae remained intact, resulting in a striate or ringed pattern (fig. 6e and k). This seems to be a result of the enzymes preferring the softer, less crystalline rings of the starches (Pérez and Bertoft, 2010; Sheets, 2016). The small granules mostly show big craters from the outside to the center, resulting in a “half-moon shape” that wanes until the grain is digested completely (fig. 6g). Potatoes show two different types of bacterial attack. Either they are digested from the hilum, then the interior is degraded completely and only the outer shell is left (fig. 6q-t) (this degradation type was described by Meireles et al., 2009), or the digestion starts from the outside. In the latter case, the granules seem to be almost intact with only the outer shell affected, which can be only observed when using different focus layers (fig. 6u-x). Mung bean starches are usually digested beginning at the mesial longitudinal cleft (fig. 7i-p). Maize granules are attacked

325 from the outside, resulting in small round pits and craters in the surface of the granules (fig. 7s-v). These differences among taxa remain even when the starches are incubated together in the mixed-starch solution (fig. 8).

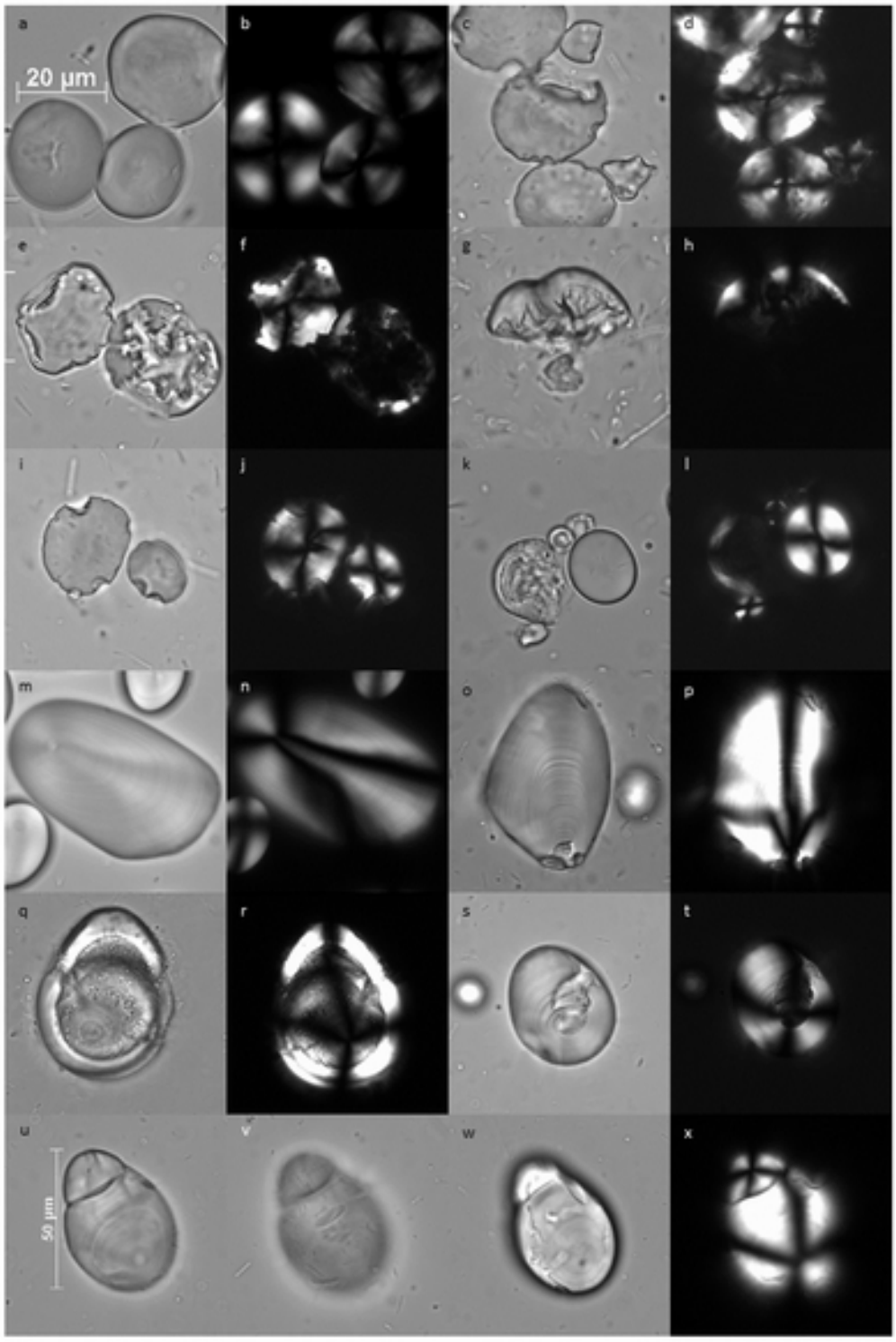

Figure 6: *Native and enzyme-damaged wheat and potato starches from the single-starch digestions. The left image of each pair is under brightfield and the right under cross-polarized light. The scale bar in a applies to all of the wheat images and that in u applies to all of the potato images. a&b: Native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

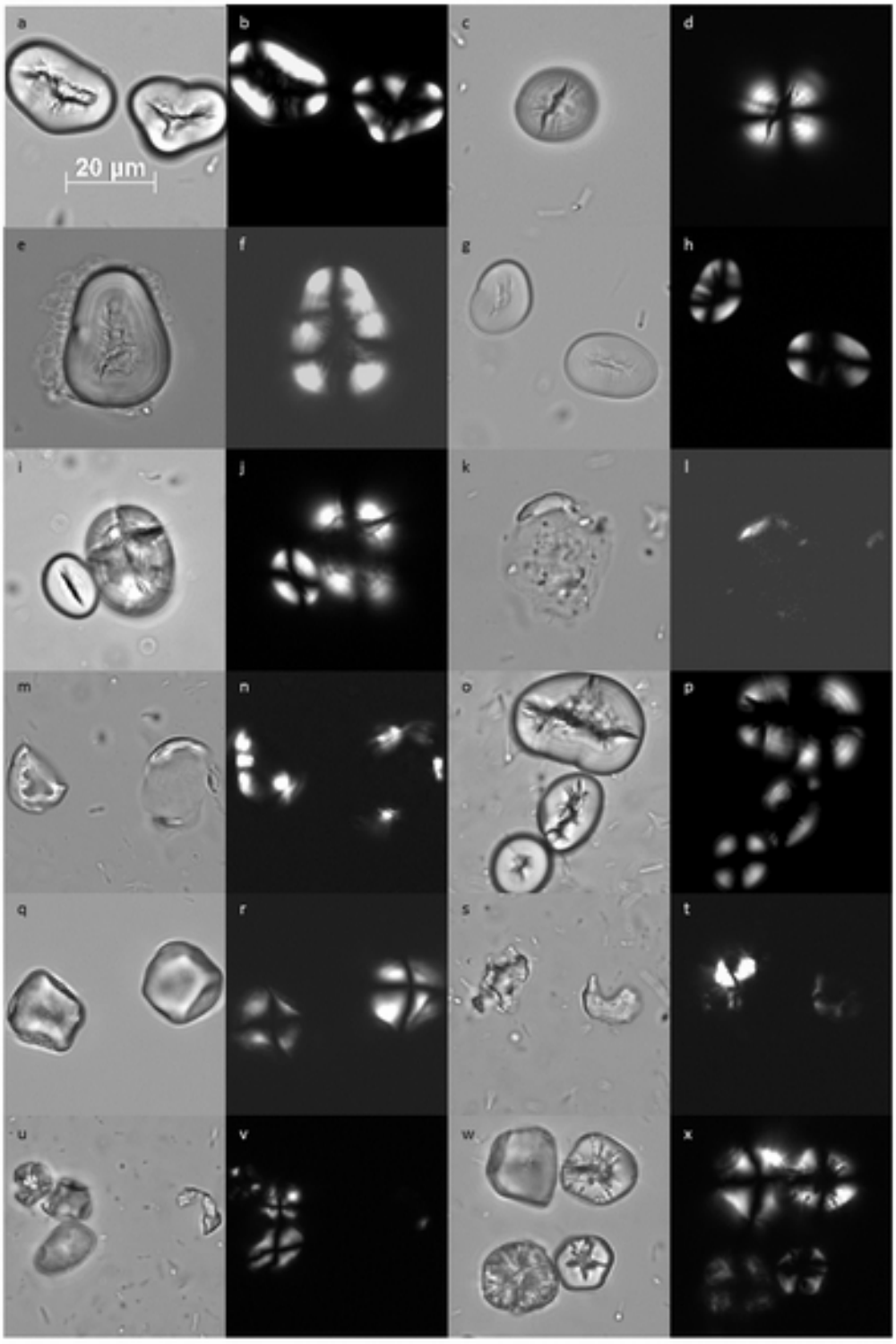

Figure 7: *Native and enzyme-damaged mung bean and maize starches from the single-starch digestions. The left image of each pair is under brightfield and the right under cross-polarized light. The scale bar in a applies to all starches. a-h: native mung bean starches (note the erratic and variable mesial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

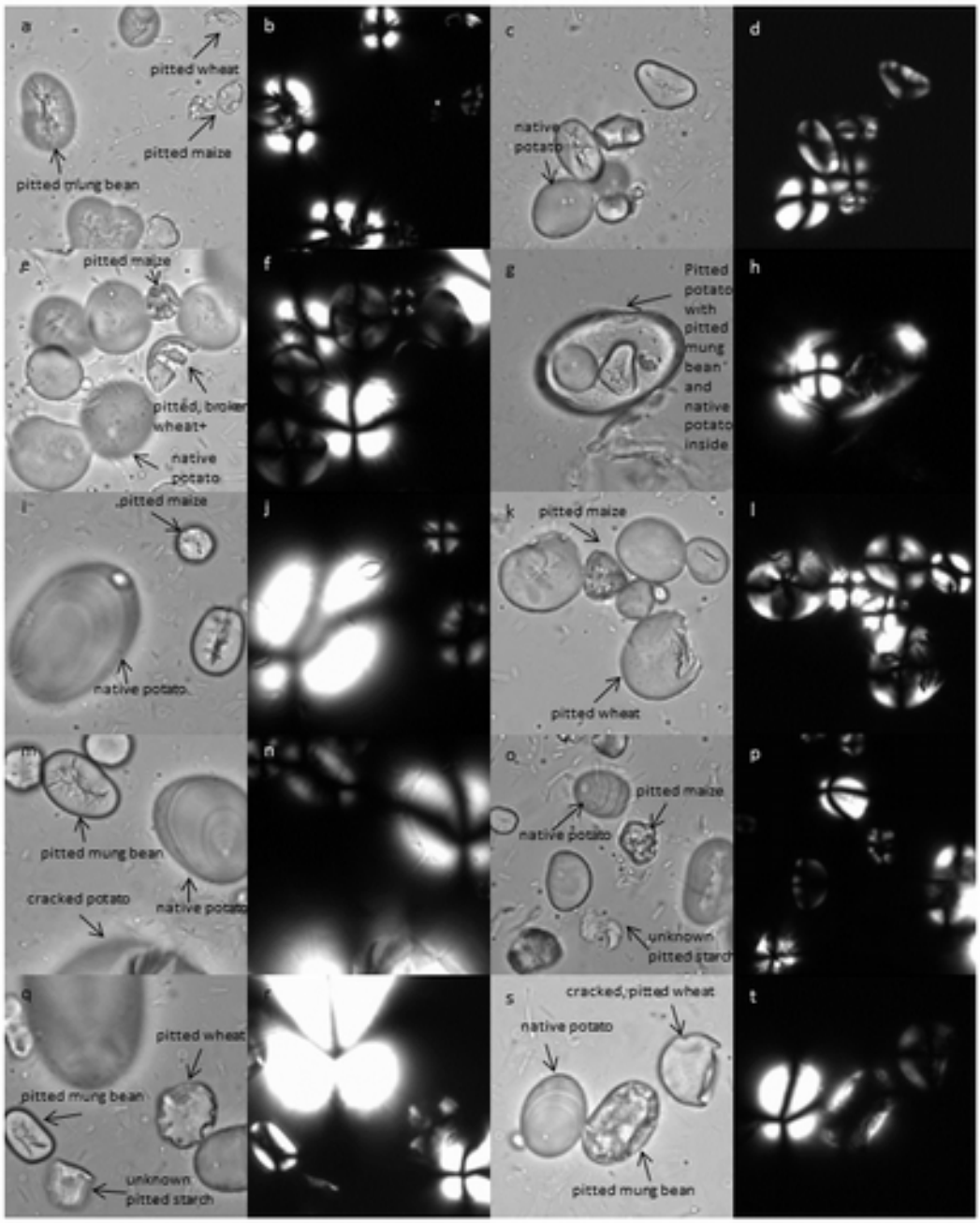

Figure 8: *Native and enzyme-damaged starches from the mixed starch digestion. The left image of each pair is under brightfield and the right under cross-polarized light. Each subfigure contains starches from several taxa.*

3.4. Bacterial digestion of big and small wheat granules

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

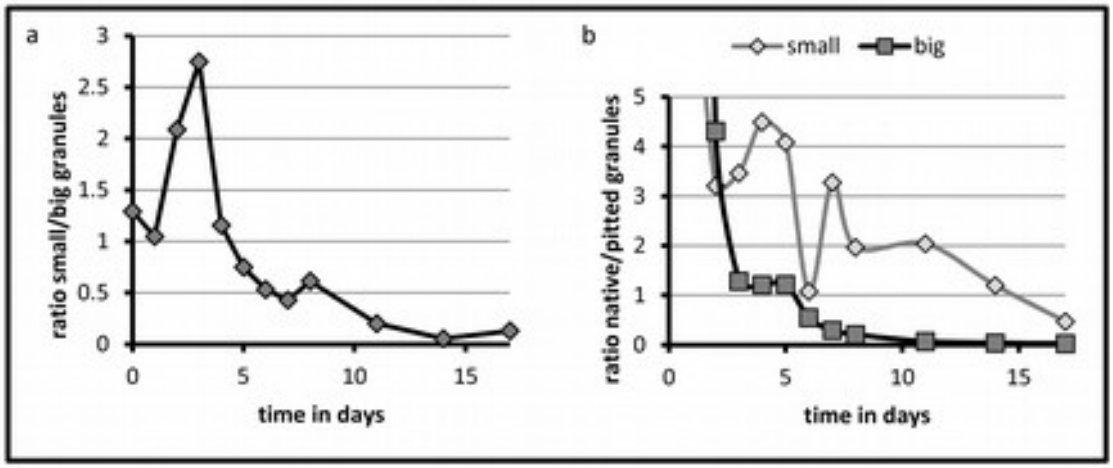

Figure 9*: Digestion of small and big wheat granules by bacterial enzymes. A) Ratio of small and big granules over time, b) proportion of pitted and native granules for small and big wheat over incubation time.*

3.5. Subjectivity in counting the different types of damage

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

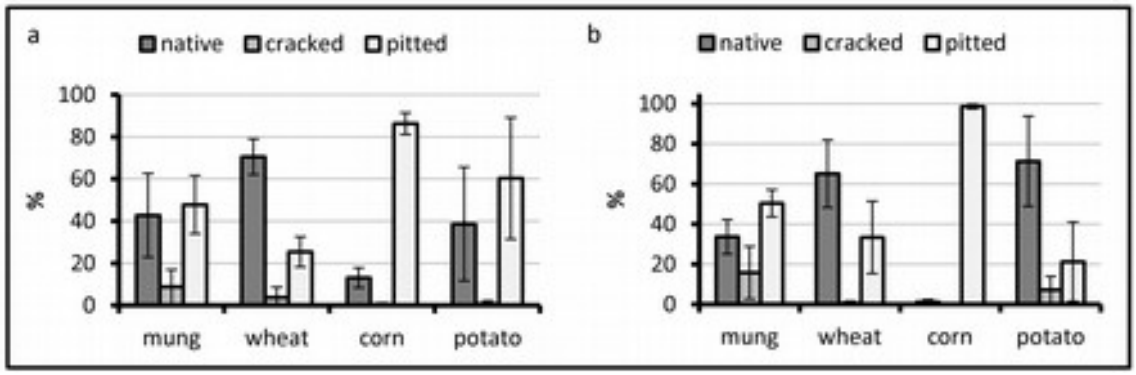

Figure 10: *Mean values and standard deviations of independent counts of the same samples by three different observers. a) Mean values and standard deviations obtained from single starch samples, b) mean values and standard deviations after three independent counts of starches from each of the four taxa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

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

3.6. Enzymatic damage on archaeological and experimental starches

In order to explore whether pitting damage could be observed on starch grains exposed to conditions more relevant to archaeology, we re-examined data collected from two other studies. For the first, we reanalyzed the starches recovered from an experiment in which starch-covered stone tools were buried and therefore exposed to native soil bacteria for two years. In this previous experiment, we had created retouched stone flakes and exposed them to one of seven treatments: raw potato, cooked potato, raw wheat, cooked wheat, raw cattail, cooked cattail or no plant (Debono Spiteri et al., 2014), with three replicates of each treatment. About 180 of these flakes were buried in seven different sites around Europe and dug up after two years. Of 1488 starches recovered from all stone flakes, 444 displayed pitting similar to that seen in this experiment (30% of the assemblage). These pitted starches were not evenly distributed throughout the sample, and instead were found in large clumps on only four flakes, with some clumps including more than 100 starches. All of the identified pitted starches came from potatoes; none of the pitted starches could be identified as wheat or cattail, though some were non-diagnostic forms. Three of the four flakes were from the same site – an agriculturally-maintained meadow. These three flakes had initially been exposed to raw potato, and comprise the three replicate treatments of raw potato for this site. All three also had native potato starches preserved, though the ratio of native to pitted was roughly 1:2. Only six of the 18 other flakes from this site showed any preservation of starches, and these were generally non-diagnostic and in very low numbers (1 or 2 on each flake). The stone flakes had

initially been heavily covered in plant material, including presumably intact cell walls, providing an extra layer of protection to the starches. This pattern was also observed in another test of buried starches (Barton, 2009). Furthermore, the inherent resistance of potato starches to enzyme damage might explain why some starches could show pitting but not be entirely removed from the record. It is also possible that the bacterial community in the meadow were not particularly well-suited to digesting potato starches. In general, we can say that pitted starches are preserved on buried stone flakes only in exceptional cases. It is likely that once bacteria begin attacking starches on a flake, they make quick work of destroying the starches entirely.

The stone flake burial study only ran for two years, and may have limited value for understanding pitted starches in deep time. We reanalyzed images collected during a previously published study of Neanderthal and early modern human dental calculus and stone tool samples (Henry et al., 2014) in order to see whether pitted starches could be recognized in archaeological samples. This study included 125 stone tools and 67 dental calculus samples from 36 individuals from 19 sites ranging in age from 8 ka to at least 130 ka and possibly up to 430 ka (the age of one sample is not firmly established). From these samples, we recovered 626 starch granules, of which ten displayed damage that is consistent with enzyme digestion (fig. 11), comprising only 1.8 % of the total assemblage. Pitted starches were found on older (c. 100 ka) and younger (c. 20 ka) material, and from all of the main geographical regions covered in the initial study (Mediterranean and northern Europe, the Near East, and central and southern Africa) (Table 2). Despite this widespread preservation, the overall extremely low number of pitted starches suggests that such damaged starches are unlikely to survive the long-term taphonomic processes that can affect archaeological assemblages. We already observed that once bacterial action begins on starches, the starches very quickly become completely digested. Even if the enzymatic action pauses, the partially-digested starches are likely more susceptible to changes in temperature, moisture, and pH.

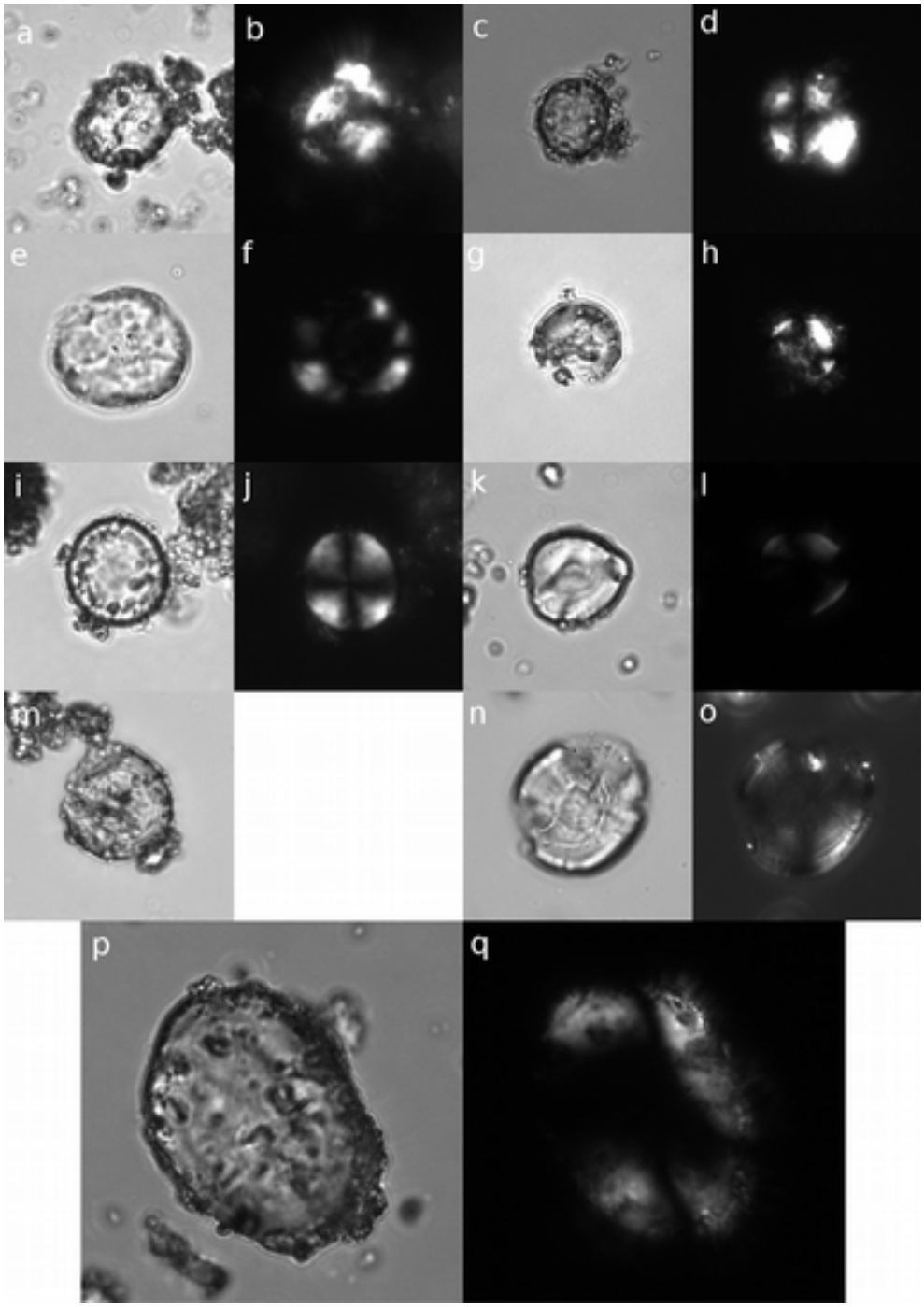

Figure 11: *Starch granules from archaeological specimens from Henry et al. (2014) showing damage consistent with enzyme digestion. The right side of each pair shows the starches in brightfield, the left under cross-polarized light. a&b: from calculus sample Blombos 8971 (SAM-AP 8971 left upper deciduous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

Table 2: Pitted starches from archaeological samples

starch / sample ID	site and age	type	number starches on sample	number of types on sample	number samples in site / level	average and range of starches in site / level	average and range of types from site / level
SK 4 used 7c	Skhul Cave level b1n: 130-100 ka	stone	13	8	5	7 (1 - 13)	4.6 (1 - 8)
SK 22 used 1a	Skhul Cave level b: 130-100 ka	stone	3	2	3	3.7 (0 - 7)	2.7 (2 - 6)
KRM1b10 1 unused 7c	Klasies River Mouth Shelter 1b: 102-98 ka	stone	20	8	5	5.4 (0 - 20)	2.8 (0 - 8)
KRM1b10 4 unused 1f	Klasies River Mouth Shelter 1b: 102-98 ka	stone	1	1	5	5.4 (0 - 20)	2.8 (0 - 8)
KRM14 11 unused 1d	Klasies River Mouth Main Cave: 102-98 ka	stone	4	3	14	2.4 (0 - 5)	2 (0 - 4)
Blombos 8971 2c	Blombos Bay Cave Layer BBC 1: 99-70 ka	calculus	2	2	2	1.5 (1 - 2)	1.5 (1 - 2)
LaFerr 1 LUM3 1c	La Ferrassie I: 74-68 ka	calculus	1	1	8	0.2 (0 - 1)	0.2 (0 - 1)
Gib 6 flat 1d	Gorham's Cave: 47-33 ka	stone	2	1	17	1.6 (0 - 12)	0.9 (0 - 5)
Spy 12B M1 25d	Spy I: 37-36 ka	calculus	>45 *	11	4	33.7 (4 - 82)	10 (0 - 21)
Ishango I15 LM1 3e	Ishango: 20 ka	calculus	4	4	6	2.7 (0 - 6)	2 (0 - 4)

** this sample included a large clump of >20 starches embedded in calculus that were not individually counted. The number of starches and potentially the number of types is therefore a minimum estimate.*

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

Our experiment clearly demonstrated that the enzyme damage to starches from particular taxon varies depending on whether starches from just that taxon or a mix of starches are available. This pattern further emphasizes the need to consider not only individual starches, but rather to look for overall patterns within an assemblage. For each of archaeological samples with pitted starches, we explored how the pattern of recovered starches compared to that on other samples from the same assemblage. Assemblages were defined as samples coming from the same site and level, and therefore represent a single group of people with similar diets, and also were from the same sedimentary contexts and subjected to similar taphonomic processes. We compared starch types rather than taxa because in many cases we were unable to identify the taxonomic origin of the archaeological starches. In some cases, several types may come from one plant species, and in other cases, one type may represent several taxa. As seen in table 2, we found that the samples with pitted starches always had several other starches, often representing different starch types. These samples were often among those with the best preservation (defined as having the most starches and the most starch types) within the assemblage. However, no sample had more than one pitted starch. Taken together, these patterns suggest that pitted starches may be preserved only in those microenvironments which are particularly conducive to general starch preservation. We cannot conclude that these pitted starches are firm evidence for biasing for or against certain taxa, however. Given our experimental data from the mixed samples, we might have expected a biased sample to have only one starch type represented on the samples which had been affected by sedimentary bacteria (i.e, all of the other starch types had been digested). In contrast, we saw that the samples with pitted bacteria were likely the ones least affected by bacterial action, with the most starches and most types preserved. The low numbers of starches on the samples with no pitted starches suggests that bacteria had completely removed the entire starch record on those samples, leaving behind a possibly less-biased assemblage on the samples with pitted starches.

4. Conclusion

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

All of these observations are relevant for understanding the preservation of starch granules in the archaeological record, and interpreting the results of starch grain analyses. While the swift digestion of starch and inter-observer variability are somewhat worrisome, our results also confirm the presence of starches that are very resistant to bacterial digestion, despite our use of environmental conditions that are the most conducive to bacterial damage (i.e., starches dispersed in a liquid suspension, high bacterial load, very warm and constant temperatures). Such growth conditions are rarely found in archaeological sites, suggesting that the rate of digestion of archaeological starches would be much reduced compared to what we document here. The finding of differential survival of starches in mixed samples strongly implies a need for caution when interpreting the relative proportion of different starches in an archaeological sample. However, the presence of starch still speaks to the presence of a particular plant type, and the recovery of pitted starches in archaeological samples can attest to the action of enzymes. The source of these enzymes, whether from salivary amylase, oral bacteria, soil bacteria, or endogenous plant amylase, is impossible to determine. The long-term survival of pitted starches is likely to occur only in

exceptional cases where the bacterial activity is arrested after the initial exposure, and should not be expected in most assemblages.

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