Journal of Archaeological Science: Reports 15 (2017) 95–108

Contents lists available at ScienceDirect

Journal of Archaeological Science: Reports

journal homepage: www.elsevier.com/locate/jasrep

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, in the > 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 resistant 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 plant taxa (wheat, maize, potato and bean) both individually and mixed together, by a mixture of unknown soil bacteria derived from local

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http://dx.doi.org/10.1016/j.jasrep.2017.07.006
Received 19 October 2016; Received in revised form 27 June 2017; Accepted 7 July 2017

'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 h 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 was 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 25,000 rpm in short bursts for 2 min. 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 ddH_2O. 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 146,432, 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, 254 nm) for 2 min 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