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Leave no stone unturned: Exploring the metaproteome of beerstone for the identification of archaeological beer production

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ABSTRACT

In archaeological contexts, identifying processes of beer production and consumption has contributed to our understanding of agriculture, labor mobilization, economic surplus, feasting, gender dynamics, social structure, tribute, community, identity and politics. Nevertheless, in the absence of pictorial representations and characteristic objects, beer brewing is difficult to identify in the archaeological record, and molecular methods are often limited by constraints of preservation and specificity. A potential target for studies of ancient beer production are residues formed during brewing activity, including beerstone, a calcium oxalate residue. Here, we apply shotgun proteomics analyses to a sample of modern beerstone to explore this residue's capacity as a marker for beer in archaeological contexts. The beerstone proteome was compared to the protein profiles of ungerminated and germinated barley to identify key proteins indicative of malted grains which may be encased by the residue. Proteins matching to barley grain (*Hordeum vulgare*) and Baker's/Brewer's yeast (*Saccharomyces cerevisiae*) were successfully identified in the beerstone. In particular, we identified hordeins, lipid transfer proteins, trypsin/α-amylase inhibitors, and protein Z, which are barley proteins abundant in proteomic characterisations of beer. In comparison to ungerminated and germinated barley grains, we find that beerstone preserves only a subset of the barley proteome, with the residue being more reflective of the final brewing product than of earlier brewing steps such as malting. Overall, we demonstrate that beerstone has potential to entrap and preserve proteins reflective of the beer-making process and identify proteins that we might anticipate in future archaeological analyses.

1. Introduction

Alcoholic beverages such as beer constitute a specialized category of food often with significant economic requirements, including surplus grain, large amounts of time and fuel, and suitable technology. Alcoholic beverages are thus imbued with economic, social, political, and ritual importance, serving varied roles not only as food and drink, but as currency, tribute, tools of labor mobilization, ritual objects, and symbols of social status (Jennings et al., 2005; Dietler, 2006; Guerra-Doce, 2015). The contexts of the production and consumption of alcoholic beverages often align with special circumstances of communal consumption, such as feasts (Hayden and Dietler, 2001; Dietler, 2006). Feasting events serve as platforms for social and political action centered around the shared consumption of foods or drinks (Hayden and Dietler, 2001; Hayden and Villeneuve, 2011). The presence of alcoholic beverages heightens the drama of the event due both to the lengthy production process and the psychoactive effects of beer consumption. In feasting contexts, alcoholic beverages become "embodied material culture" (Dietler, 2006:229), and the act of offering and consuming creates a reciprocal relationship between the host and the guest which often carries corresponding social and political returns (Mauss, 1954; Turner, 1968). The role of alcoholic beverages is thus innately tied to commensal occasions in which cultural values and social relationships can be reinforced and challenged. Recognizing the archaeological production and

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consumption of beer — defined here as a fermented liquid, alcoholic product of germinated grain — can thus provide important insights into ancient systems of agriculture, foodways, economy, society, beliefs, and politics (see Table 1).

Despite the availability of numerous indicators for beer production and activities in archaeological contexts, ancient beer identification and reconstruction remains challenging (Stika, 2011). Textual and iconographic depictions of beer making and drinking emphasize the central role of beer in economic and political contexts, though these forms of evidence are not widespread (Katz and Voigt, 1986; Hartman and Oppenheim, 1950; Homan, 2004; Michel et al., 1993). Archaeological features and ceramics can attest to beer production and consumption, particularly in the identification of large-scale beer production (i.e. dedicated breweries). Large open-mouthed pots, or 'vats', funnels, and vessels with perforated bases, for example, have been cited as material evidence for beer production, while contextual features interpreted as hearths, ovens, and malting floors further support interpretations of brewing activity (Arthur, 2003; Hayashida, 2008; Dietrich et al., 2012; Valamoti, 2023). Microbotanical and macrobotanical remains adhering to vessels or identified in brewing contexts have been used as further evidence for brewing activity (Geller, 1993; Stika, 1996; Wang et al., 2016; Valamoti, 2018; Heiss et al., 2020), though these efforts are often limited by recovery and preservation. Taxonomic determinations of botanical remains have nonetheless transformed our understanding of the diverse ingredients and processes of beer brewing. For example, using criteria developed to distinguish damage patterns on starch grains caused by mashing and fermentation, Wang et al. (2016, 2017) present microbotanical evidence for millet/barley beer associated with Yangshao ceramics from Mijiaya, China; and Heiss et al. (2020) uses the thinning of the cell walls in the cereal grain aleurone layer —alongside additional archaeological evidence for brewing— as a marker for malting in Predynastic Egypt and Late Neolithic Central Europe.

Multiple methods of chemical residue analysis, including the Feigl spot test, high-performance liquid chromatography (HPLC), ion chromatography, and fourier-transform infrared spectrometry (FTIR), have been employed to identify oxalate ions, proposed as markers of beer production, in archaeological ceramics (for example, Michel et al., 1993; McGovern et al., 1999; McGovern et al., 2005; Otto, 2015). The earliest reported identification of beer in the Middle East, for example, is based on a positive identification of oxalate ions on a Late Uruk ceramic sherd from Godin Tepe in Iran (Michel et al., 1993). Diagnostic compound markers for beer beyond oxalate ions have also been proposed when present in tandem, and include hydrocinnamic acid, methoxycinnamic acid, benzoic acid, butanedioic acid, pimelic acid, suberic acid, azelaic acid, squalene, pyrrolo [1,2-a]pyrazine-1,4-dione, and glycerol (Perruchini et al., 2018). Nonetheless, existing chemical analyses of beer face significant challenges with pre- and post-burial contamination, and calcium oxalate specifically is present in a variety of plants unrelated to beer production, as well as within soil (Cramp and Evershed, 2015; Whelton et al., 2021). These issues are further compounded by a lack of standardization for the identification and analysis of organic compounds from archaeological material more generally (Whelton et al., 2021). Additional analytical techniques are thus required to better identify brewing activity and capture variations in beer production in the archaeological record. The presence of specific organic compounds (i.e. lipids and organic acids) absorbed in pottery and pottery residues, for example, have been used to suggest possible beer making and drinking in Early Celtic Central Europe (Rageot et al., 2019). In addition, a recent study by Farag et al. (2019) applied metabolite analyses to vat residues from Hierakonpolis, Egypt, demonstrating the contribution of various compounds in beer's constitution and advancing our understanding of the chemical profile of archaeological beer. With the application of multiple chemical methods, archaeologists can thus seek to build comprehensive recipes for ancient beer.

Table 1
Description of beerstone and grain samples analyzed using metaproteomics.

Sample ID	Sample Description
VatBS	Beerstone from fermentation vat
0 A	Ungerminated barley grain
0 B	Ungerminated barley grain
5 A	Germinated barley grain (5 days into germination process)
5 B	Germinated barley grain (5 days into germination process)
Blank	Extraction blank control

1.1. Beer production and the formation of beerstone

At its simplest, beer-making involves the fermentation of cereal grains to produce alcohol. Modern beer production is marked by five main stages: malting, mashing, boiling, fermentation, and maturation (Fig. 1). In the primary stage, grain is germinated (sprouted/malted), and then dried to create storable malt. Malt is milled or ground before being mixed with heated water in a process known as "mashing". The mash is strained, and the resulting liquid, known as "wort", is then boiled. Additional plants, such as *Myrica gale* and hops (the latter in more recent times), can be added for various purposes, impacting flavour or increasing the shelf-life of brews. After the mixture has cooled, yeast is loaded to initiate fermentation. The beer is then aged, strained, stored, and prepared for consumption through the process of maturation. Individual brewers often direct variation in the process, with small changes creating different flavours, colours and alcohol strengths. While ancient beer-making may involve different processes, it is important to be aware of modern practices in this study, as each step will influence the protein composition of the final product and of the beerstone.

Beerstone is a mineralized residue that forms on the interior of vessels in the latter stages of the beer-making process: fermentation and maturation. Beerstone is composed primarily of calcium oxalate, formed through interactions between hard minerals in water and proteins from the grain (Vinkler and Cserhati, 1969; Johnson, 1998). Beerstone is not purely mineral in composition. The organic fraction is primarily made of hard plant resins and proteins precipitated by tannic acid, which act to bind the calcium oxalate to surfaces (Vinkler and Cserhati, 1969; Johnson, 1998). Due to the durability of its mineral structure, beerstone thus has significant potential to preserve into historical and archaeological timescales, and potentially provides a stable mineral matrix to which ancient biomolecules can bind.

Here we apply metaproteomic analysis to modern beerstone for the identification of proteins indicative of beer production. Proteomics has been successfully applied to archaeological ceramics, vessel contents and residues for the identification of food-derived proteins (Solazzo et al., 2008; Hong et al., 2012; Shevchenko et al. 2014, 2018; Yang et al., 2014). Limescale, a mineralized calcium-based residue adhering to ceramic vessel interiors, has demonstrated particular success in preserving ancient proteins related to food preparation (Hendy et al., 2018b; Evans et al., 2023). Due to its calcium oxalate structure, we hypothesize that beerstone has similar potential to preserve ancient proteins as a non-soluble residue which binds to vessels of fermentation and maturation. Characterizing proteins preserved in ancient beerstone residues could identify ingredients — such as cereal grains and microorganisms — utilized in beer production, leading to a richer understanding of the diversity and manufacture of this foodstuff.

The aim of our study is to characterize the proteome of modern beerstone, to identify which proteins may become entrapped in this residue and survive to indicate beer-making processes, and to anticipate what proteins might then be preserved in archaeologically analogous residues. We applied shotgun proteomics to a modern sample of beerstone collected from a fermentation tank in current use. Working with the hypothesis that the beerstone would contain proteins specific to (or more abundantly expressed) in germinated grains, we also compared the barley proteins identified in the beerstone to the proteome of

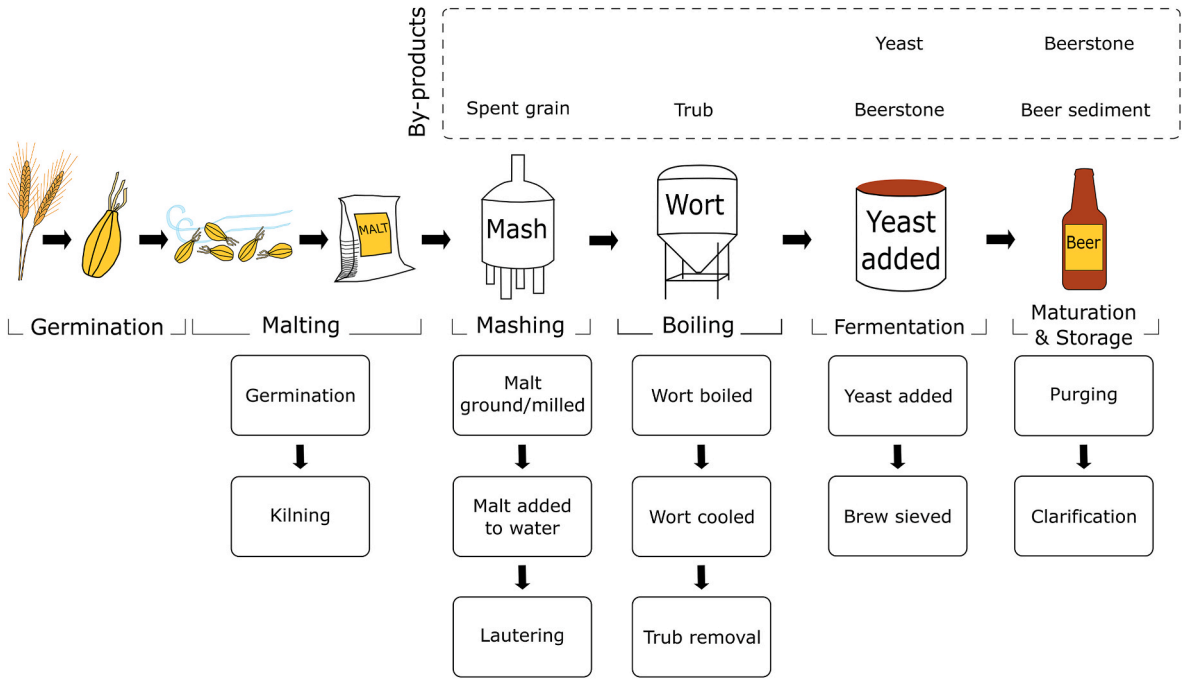

Fig. 1. A depiction of the modern beer production process and its byproducts.

ungerminated (collected after 0 days of germination) and germinated barley grains (collected after 5 days of germination).

2. Materials and methods

2.1. Sample collection

A sample of modern beerstone was obtained from York Brewery, York, UK in 2019. As beerstone can act as a reservoir for bacteria which may negatively affect the taste and composition of brews, breweries have dedicated protocols to both minimize beerstone formation and to remove beerstone from equipment. At York Brewery, vats are chemically cleaned after use to remove beerstone. The sample of beerstone used in this study ('VatBS') was collected from a fermentation vat that had been rinsed with water prior to chemical cleaning. The vat had been used to brew Guzzler Pale Ale which is predominantly made from pale malt supplied by Thomas Fawcett & Sons, Ltd. (Castleford, UK), who germinate malt for 5–7 days using floor malting (a traditional method where grains are spread across an expansive floor). At York Brewery, pale malt is mashed at approximately 67 °C, sieved, and then boiled at 105–112 °C within a 70-min period. Hops are added before and after the boiling. The wort is cooled and emptied into a vat. Yeast is loaded to ferment the wort for approximately three days. Beerstone forms primarily in these latter stages, during wort boiling, cooling, fermentation, and maturation.

The ungerminated and 5-day germinated two-row hulled barley (*Hordeum vulgare* subsp. *vulgare f. distichon*) grains were provided by Heinrich Durst Malzfabriken, Bruchsal-Heidelsheim, Germany, under the framework of the PLANTCULT Project (Valamoti et al., 2017). The grains were then oven-dried. Two whole grains from each state were selected for analysis to capture variation between individual barley grains. Experimental charring followed by morphological and microscopic analysis of the same set of these grains has been previously published by Heiss et al. (2020).

2.2. Protein extraction and sequence characterization

The previously dried barley grains were frozen, freeze-dried, and pulverized with a mortar and pestle. 18 mg of the beerstone sample was demineralised in 500 mL of 0.5 M EDTA (pH 8) and concentrated using a 30 kDa Microcon filter unit. 50 mg of the powdered barley grain samples, and 100 µL of the beerstone sample in EDTA, were put into a lysis buffer of 1 M DTT, 250 µL of 1 M Tris/HCl, 500 µL of 20% SDS, and 1500 µL of ultrapure water. Filter-aided sample preparation (FASP) was used for protein extraction, following the protocol outlined by Wiśniewski et al. (2009) and Jeong et al. (2018). Proteins were solubilized and bonded to a 30 kDa Microcon filter unit for filtration. Cysteines were alkylated with 100 µL of 0.05 M iodoacetamide (IAA) solution. Proteins were digested with 0.04 µg/µL trypsin before being desalted using C18 StageTips (made from Empore C18 SPE disks). Peptide solutions were dried using a vacuum centrifuge before analysis using an Orbitrap Fusion LC-MS/MS mass spectrometer at the University of York.

The samples were diluted 10-fold and ran over a 3-h acquisition. The nanoLC system was interfaced with an Orbitrap Fusion Tribrid mass spectrometer (Thermo) with an EasyNano ionization source (Thermo). Positive ESI-MS and MS 2 spectra were acquired using Xcalibur software (version 4.0, Thermo). Instrument source settings were: ion spray voltage, 1900 V; sweep gas, 0 Arb; ion transfer tube temperature; 275 °C. MS 1 spectra were acquired in the Orbitrap with: 120,000 resolution, scan range: m/z 375–1500; AGC target, 4e 5; max fill time, 100 ms. Data dependent acquisition was performed in topN mode selecting the 12 most intense precursors with charge states 1. Easy-IC was used for internal calibration. Dynamic exclusion was performed for 50s post precursor selection and a minimum threshold for fragmentation was set at 5e 3. MS 2 spectra were acquired in the Orbitrap with: 30,000 resolution, max fill time, 100 ms., HCD; activation energy: 32 NCE.

2.3. Data analysis

MS/MS ion database searching was performed with MaxQuant (release 2022–08) (Tyanova et al., 2016). The barley grain samples were searched against the barley proteome (*Hordeum vulgare* subsp. *vulgare*, Uniprot proteome: UP000011116) and the contaminant database previously published in Evans et al., (2023) for use with archaeological samples. The beerstone sample was searched against the same contaminant database and Swiss-Prot (release 2022–08) to identify the diversity of proteins that might be entrapped within this substrate. Precursor mass

tolerance was set to 20 ppm and product mass tolerance was set at 4.5 ppm. Carbamidomethyl of cysteine was set as the fixed modification, while oxidation (MP), deamidation (NQ), and acetyl (N-term) were selected as variable modifications. The fully tryptic searches – allowing for a maximum of 2 missed cleavages – were performed against a decoy database to estimate protein false discovery rates (FDR). Protein results were filtered to an FDR of 1% and containing a minimum of two distinct peptides matching to different regions of the protein. Protein families matching to entries within the contaminant database were excluded from further analysis. Protein expression data (tissue or cell specificity) were retrieved from UniProtKB where available. Taxonomic identifications of peptides were investigated using Unipept for lowest common ancestor analysis (Mesuere et al., 2015) and for barley and yeast identifications STRING v. 11.5 (Szklarczyk et al., 2019) was used to analyze protein–protein interactions and proteome function using gene ontology (GO) annotations and expression profiles. Raw and processed data for the barley grain samples (0 A, 0 B = ungerminated, and 5 A, 5 B = germinated) and the beerstone sample (VatBS) were deposited to ProteomeXchange Consortium (dataset identifier: PXD053177) through MassIVE (University of California San Diego, CA, USA).

3. Results

3.1. Barley grains

We analyzed ungerminated (0 A/B) and 5-day germinated barley grains (5 A/B), performed in duplicate. After quality filtering, 386 protein groups were identified in sample 0 A, 212 protein groups were identified in 0 B, 495 protein groups were identified in 5 A, and 547 protein groups were identified in 5 B. Generally, fewer protein groups were identified in the ungerminated barley grains (0 A/0 B, N = 598) when compared to the germinated (5 A/5 B, N = 1042) dataset. We then explored proteins that were uniquely identified only in either the ungerminated or germinated grain, detecting 29 in the former and 166 in the latter (Fig. 2). An analysis of the ungerminated and germinated datasets through STRING (Kanehisa and Goto, 2000; Kanehisa et al., 2023) suggests a significant difference in KEGG metabolic pathways for both grain types (Supplementary Fig. 1). The RNA transport KEGG pathway (map03013) was the main pathway represented in the ungerminated dataset (7 out of 290 proteins). The citrate cycle (TCA cycle) (map00020), pyruvate metabolism (map00620), proteasome (map03050), glutathione metabolism (map00480), and carbon metabolism (map01200) were the KEGG pathways identified in the germinated dataset, demonstrating more recognized metabolic activity in the germinated samples. The observed pattern is supported by existing proteomic studies of barley grains during germination (Bobálová et al., 2010; Qin et al., 2021).

3.2. Beerstone

A total of 51 protein families were identified in beerstone once potential contaminant proteins were removed. The most represented taxonomic groups were yeasts, represented by the genus *Saccharomyces* (17 protein groups), followed by barley (*H. vulgare*) (9 protein groups). Using UniPept metaproteomics analysis to identify the taxonomic assignment of individual peptides, peptides were also assigned to higher taxonomic orders, such as Dikarya N = 4, Saccharomycetaceae N = 7, Saccharomycetales N = 1, and Saccharomyceta N = 2; and Triticeae N = 1 (Fig. 3). No proteins from hops (*Humulus lupulus*) were identified.

3.2.1. Saccharomyces proteins in the beerstone

The *Saccharomyces* proteins in the beerstone derive from the added yeast for fermentation. The *Saccharomyces* genus encompasses many species, including *S. cerevisiae*, also called Baker's or Brewer's yeast. A functional analysis of the identified yeast proteins in the beerstone presents a characteristic pattern of yeast metabolism, in which carbohydrates are transformed into ethanol and carbon dioxide (CO_2) (Supplementary Fig. 2). Detected proteins glyceraldehyde-3-phosphate dehydrogenase 1 (P00360), enolase 2 (P00925), phosphoglycerate kinase (P00560), and phosphoglycerate mutase 1 (P00950), among others, are involved in the biological process of glycolysis, a subpathway of carbohydrate degradation that converts glucose into pyruvate. Pyruvate proteins, including pyruvate decarboxylase isozyme 1 (P06169) and pyruvate kinase 1 (P00549), are produced from glycolysis and involved in the conversion of pyruvate to acetaldehyde and CO_2 during fermentation. Alcohol dehydrogenase 4 (P10127) reduces the available acetaldehyde to ethanol. Magnesium-activated aldehyde dehydrogenase, cytosolic (P54115) is involved in the pathway of ethanol degradation.

3.2.2. Barley proteins in the beerstone

When searched against Swiss-Prot, 9 protein families assigned to *H. vulgare* were identified in the beerstone, including trypsin inhibitor CMe (P01086), serpin-Z4 (P06293), B1-hordein (P06470), non-specific lipid-transfer protein 1 (LTP 1) (P07597), alpha-amylase inhibitor BDAI-1 (P13691), alpha-amylase inhibitor BMAI-1 (P16968), probable non-specific lipid-transfer protein (LTP) (P20145), alpha-amylase/trypsin inhibitor CMb (P32936), and serpin-Z7 (Q43492) (Supplementary Table 1). Serpin-Z4 is noted by UniprotKB to be tissue specific to the barley embryo and endosperm. Serpin-Z7 is predominantly found in the endosperm, though is present in lower quantities in the embryo and roots (Roberts et al., 2003). B1-hordein, alpha-amylase inhibitors BDAI-1 and BMAI-1, alpha-amylase/trypsin inhibitor CMb and trypsin inhibitor CMe are similarly specific to the endosperm, with trypsin inhibitor CMe notably absent in the embryo, aleurone, coleoptile, roots, and leaves. LTP 1 and probable non-specific lipid-transfer proteins are highly specific to the aleurone layer of developing and germinating seeds (Skriver et al., 1992).

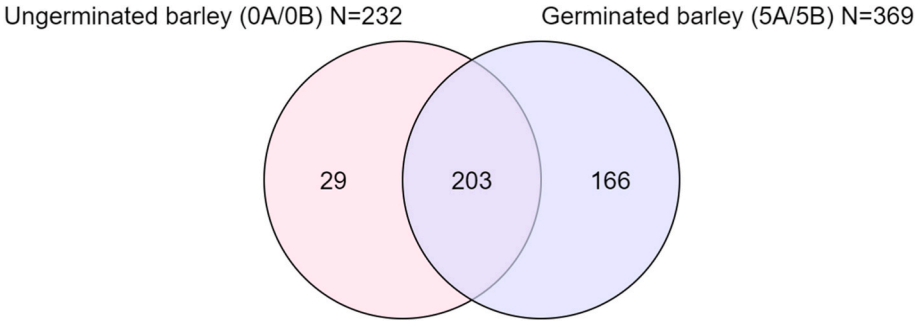

Fig. 2. Venn diagram depicting the number of shared (203) and unique (29 and 166, respectively) barley protein groups between the ungerminated (0 A/0 B) and geminated (5 A/5 B) datasets.

Fig. 3. Taxonomic composition of the beerstone (VatBS sample), limited to the rank of kingdom. Created with Unipept (Mesuere et al., 2015).

Following this wide search using the Swiss-Prot database, the beerstone sample was also searched using MaxQuant against the *H. vulgare* subsp. *vulgare* proteome (Uniprot: UP000011116) to allow comparison between the barley grain proteome and the barley proteins present in the beerstone sample. We aimed to evaluate whether the beerstone contained a proteome that might specifically reflect germinated grain. Searching against the *H. vulgare* subsp. *vulgare* proteome, only 10 barley protein families were identified in the beerstone: AAI domain-containing protein, D-hordein, acidic protein, vignain-like protein, elongation factor 1-alpha, non-specific lipid transfer protein, SERPIN domain-containing protein (which encompasses Serpin-Z4, Z7, and ZX), predicted protein, hordoindoline-B1, and dehydrin 7 (Supplementary Table 2). Eight of the identified barley proteins were also detected in both ungerminated and germinated grain samples (Supplementary Table 2), with two protein families shared exclusively between the germinated barley grains and the beerstone: dehydrin 7 (Uniprot: T1TEB9), a stress response protein; and vignain-like (Uniprot: A0A8I6XRW9), a protein involved in proteolysis and not specific to germination. The overall protein composition of the beerstone is thus more similar to the germinated dataset than the ungerminated dataset, however the number of barley proteins identified in the beerstone are few. Moreover, the barley proteins identified in the beerstone sample do not appear to be specific to germination and are instead generally common in barley and other cereal grains.

4. Discussion

Our study sought to explore protein signatures of beer brewing through the proteomic analysis of beerstone, a residue by-product of the brewing process. This substrate serves as an analogy to potential residues adhering to archaeological artefacts that may indicate beer-making. In summary, our analyses identified proteins matching *Saccharomyces* yeast and *H. vulgare* in beerstone, indicating the survival of these key indicators of beer-making. Though the analyses did not specifically detect barley proteins indicative of the use of germinated grains, the overall beerstone proteome does share similarities with previous analyses of beer proteomes (Picariello et al., 2012; Colgrave et al., 2013). Every barley protein identified in our Swiss-Prot search of the beerstone was previously reported in Colgrave et al.'s (2013) syntheses of reported barley proteins in beer. Moreover, the presence of a strong signature of yeast together with the barley could indicate a vessel's use for beer-brewing, though analyses of archaeological beerstone are necessary to confirm. Here, we discuss the palimpsest of processes impacting the detection of barley and yeast proteins in beerstone, and the potential of the biomolecular analysis of beerstone to indicate past beer-brewing in archaeological contexts.

4.1. The preservation of barley proteins in beerstone

The identification of barley proteins in beerstone is key for ascribing the residue to barley-based beer-making, as opposed to other kinds of foodcrust or calcium mineral build-up, such as from dairy products (Hendy et al., 2018b; Evans et al., 2023). In comparison to barley grain proteomes retrieved in this and previous studies, we recovered relatively few barley proteins from the beerstone sample. Nevertheless, our identifications were consistent with the known proteomic composition of beer (Picariello et al., 2012; Colgrave et al., 2013) and reflect a palimpsest of the beer making process, described in detail below. The barley proteins identified in the beerstone mainly function in seed storage and plant defense and are not specific to grain germination. Protein Z isoforms (including serpin-Z4 and serpin-Z7), detected in the beerstone sample, are some of the most important barley proteins, alongside trypsin/α-amylase inhibitors, lipid transfer 1 (LTP1) and hordeins, that are regularly identified in beer (Bobálová et al., 2010). Chemical (including post-translational modification) and physical (resistance to temperature, degree of solubility, etc.) attributes of hordeins, LTP1, trypsin/α-amylase inhibitors, and protein Z, enhance their ability to survive the brewing process (van Nierop et al., 2004; Perrocheau et al., 2005).

Our study focuses on protein identifications and not on the detection of post-translational modifications, but it is worthwhile noting that proteolysis, glycation, and glycosylation have been identified as common post-translational modifications (PTMs) created during the brewing process, particularly during malting (Kerr et al., 2021). Post-translational modifications refer to chemical alterations that occur in proteins due to a myriad of processes and can influence their detection in the proteome and serve as an indicator of preservational quality. Glycation, for example, of protein Z (serpins) and lipid transfer proteins during malting produces Amadori products (Bobálová et al., 2010). These stable products of glycation may prevent lipid transfer protein precipitation during wort boiling, and result in the survival of lipid transfer proteins into the final product (Perrocheau et al., 2006). The presence of both these proteins within the beerstone, is thus likely a result of them having undergone a series of protein modifications, including glycation, in earlier stages of the beer-making process that resulted in their survival to the final product. A future analysis focusing on protein glycation and modification could potentially enrich the detection of beer-derived proteins.

Enzymes involved in the malting and mashing process may also impact the preservation of barley proteins in beerstone. The aleurone layer of barley grain hosts the major hydrolytic enzymes required for germination, including proteases and amylases (Holopainen-Mantilla, 2015). These proteolytic enzymes degrade proteins of the endosperm during malting (Klose et al., 2010), reducing the chance of these proteins surviving into the final product of the beer-making process. During mashing, active enzymes also work to degrade many proteins, while proteins with high molecular weights are prone to precipitation (Klose et al., 2010).

The methods used in beer production, including filtering, the degree of lautering (separating the wort liquid from the grain), the temperature of boiling, and the treatment of hot and cold breaks, will also affect the protein composition of both the final product and, presumably, of the beerstone. For example, during wort boiling, proteins coagulate and separate into wort and trub (i.e., coagulated protein precipitate). Trub before cooling, known as hot trub or hot break, is dominantly proteinaceous, while cold break trub, which forms after the brew has been cooled, contains protein-tannin complexes. Iimure et al. (2012) investigated proteome changes in sweet wort, boiled wort, and trub, and

found higher proportions of LTP1, among other proteins, in the boiled wort than in the trub. Proteins that remain in the trub will be removed from the brew and thus be underrepresented in the final product. Similarly, lautering, or the sieving of the mash to separate the spent grain from sweet wort, removes significant portions of proteins originating from the malting process (Klose et al., 2010). As the brewing process involves several heating steps, heat-stable proteins are more likely to be present in the final product. Hordeins, lipid transfer proteins, trypsin/α-amylase inhibitors, and protein Z (serpin Z4 and Z7) are disulfide-rich and thus more resistant to high temperatures (van Nierop et al., 2004; Perrocheau et al., 2005).

It is worthwhile noting, however, that our results and comparisons are focused on modern beer-brewing methods, aspects of which may not have been practiced in past beer production. Nevertheless, the proteins that are reflected in the beerstone are a palimpsest of the complex physical, chemical, and metabolic factors that are an innate part of the brewing process. Together, our results suggest that beerstone does not entrap a complete barley proteome, but instead, represents a small subset of proteins from germinated barley and is overall more similar to a proteome of beer as the end product.

4.2. Yeast proteins: Is yeast a marker for beer identification in archaeological samples?

In the beerstone, our analysis yielded 17 *Saccharomyces* proteins largely representative of yeast anaerobic metabolism, in which sugar is converted into alcohol. Yeast is added to cooled wort as the primary agent of fermentation. As the beerstone sample was collected from a fermentation vat, the presence of yeast is thus expected, but the degree to which it would be entrapped, and which proteins would be detected, was unknown.

In general, the yeast proteins identified in our beerstone dataset (17 proteins matching to *Saccharomyces* and 7 proteins matching to Saccharomycetaceae) align with previous studies reporting brewing yeast proteins present in beer (Kobi et al., 2004; Lodolo et al., 2008; Picariello et al., 2012; Colgrave et al., 2013). Previous studies of the yeast protein in beer have identified four main proteins of yeast that survive into the final product: enolase, triosephosphate isomerase (TPI), thioredoxin (y-TRX2p), and yeast phosphorelay protein (Ypd1) (Perrocheau et al., 2005; López-Villar et al., 2006; Iimure et al., 2010). In our dataset, only two of these four proteins were identified: enolase 1 and enolase 2, and triosephosphate isomerase. These glycolytic enzymes are released as a product of yeast cell damage, likely caused by the brewing process (López-Villar et al., 2006; Iimure et al., 2010). The stress caused to yeast cells during brewing could be responsible for the absence of y-TRX2p (and likely other proteins) in our dataset, though the overall concentration of y-TRX2p in the brew and the expression level of the protein are also influential factors (Iimure et al., 2010).

In our study, yeast with specific characteristics desirable to the brew was deliberately added, however it is important to note that fermentation from yeast can also occur spontaneously from yeasts and microbes in the local environment (such as in the making of Lambic sour beer; Spitaels et al., 2014), or from vessel reuse, the addition of fruits/honey with natural yeast blooms, insects (such as wasps and bees) harboring yeasts, and the introduction of human saliva (Homan, 2004; Jennings et al., 2005; Hayashida, 2008; Guerra-Doce, 2015; Stefanini et al., 2012). The *Saccharomyces* genera includes natural species, as well as those altered or domesticated by humans (Scannell et al., 2011). *S. cerevisiae* is most commonly used in the fermentation of wine and beer with specific strains imparting particular flavours or properties. Despite the predominance of *S. cerevisiae*, other species of *Saccharomyces* are also actively used in the fermentation of beverages, including *S. bayanus* (for cider and wine) and *S. pastorianus* (for lager beer) (Naumova et al., 2005; Lodolo et al., 2008; Nakao et al., 2009; Sicard and Legras, 2011), as well as other genera such as *Brettanomyces* (Colomer et al., 2019).

Saccharomyces proteins have been successfully extracted from archaeological material – such as desiccated coprolites from Hallstatt, Austria (Maixner et al., 2021) – and have contributed to identifications and characterizations of archaeological beer. The detection of yeast proteins as markers for ancient brewing, however, is complicated by the ubiquity of yeast in many environments (Anderson et al., 2018; Stefanini et al., 2012) and the fact that we remain ignorant of the specific yeast species utilized in ancient brewing. Certainly, evidence for beer is likely to contain yeast; however, it cannot be said that the identification of *Saccharomyces* proteins alone can be used as an exclusive proxy for brewing activity. As the authentication of ancient proteins is an issue of particular concern in the field of palaeoproteomics (Hendy et al., 2018a), the identification of yeast proteins in archaeological contexts must be particularly subject to scrutiny with regards to their endogeneity. Additional environmental controls assessing indicators of protein diagenesis would also assist in protein authentication. Further work must be conducted to better understand and capture variation in yeast proteome signatures in modern, historical, and ancient beerstone residues.

4.3. Beerstone is a viable substrate for the biomolecular detection of brewing

Our study confirms that beerstone indeed entraps proteins from beer-making (barley and yeast), but protein preservation is strongly influenced by myriad processes involved in brewing beer. Our results indicate that proteins from various aspects of the beer-making process survive into the fermentation stage (the stage in which beerstone is formed in the vat) and are entrapped within the beerstone, offering a unique insight into the proteomic composition of the brew when the liquid component is no longer accessible. Nevertheless, we also note that some known ingredients, such as hops and wheat, were not detected in the beerstone and that the beerstone does not necessarily indicate the use of *malted* barley grains.

While beerstone is common in modern brewing contexts and has been reported in archaeological contexts, the scale of its presence across different contexts, time periods and object-types is not well understood. McGovern (2009) suggests that ancient beer-makers used the matrix of ceramics, as well as grooves in these vessels, as a means to control the formation of beerstone. The absorption of beerstone into the ceramic matrix would increase the likelihood of its survival and provide a source of material that can be targeted for proteomic analysis. Previous studies claim to have identified samples of archaeological beerstone, initially describing the residue as “pale” and “yellowish” (e.g. Michel et al., 1993), while others assume beerstone absorbs into the ceramic matrix (e.g., Perruchini et al., 2018) and is thus invisible to the naked eye. A further example of these residues are those observed on stone troughs from Pre-Pottery Neolithc Göbekli Tepe (Dietrich et al. 2012, 2020).

As with any palaeoproteomic study, proteins from archaeological beerstone will be subject to diagenesis and potential contamination from the environment or handling. Nevertheless, beerstone, as a mineral construct has significant potential to protect the beer proteome from environmental contamination and from taphonomic degradation, as has been noted with limescale on ceramics (Hendy et al., 2018b; Evans et al., 2023). The resulting protein composition could provide unique insights into beer recipes, including the primary grain(s) used in brewing (millet, barley, wheat, corn, sorghum, etc.) and even the type of yeast used.

We also note, however, that there are significant taphonomic factors which will hamper efforts to identify original ingredients and beer ‘recipes’. For example, in our study there is an absence of proteins from hops, which could be due to the low protein content of hops relative to barley grains, or to diagenetic factors involved in the brewing process. As described above, beer-making steps such as boiling and filtering will substantially alter the proteome as beer is transformed from ingredients to final product. Proteomic analyses of archaeological beerstone and additional studies of modern and historic beerstone, are thus required to better understand how taphonomy and the brewing process impact the

beerstone proteome. Moreover, while the beerstone proteome as reported here is proposed as a potential marker for brewing, it remains essential to assess proteomic data alongside other archaeological evidence for beer to create more informed identifications of past brewing activity.

5. Conclusion

Beer is a hugely significant beverage, today and in the past, with its preparation and consumption imbued with social, cultural and economic significance. Using shotgun proteomics we recovered barley and yeast proteins from modern beerstone, indicating that the beerstone can retain a signature of beer production, with the proteome more reflective of the final brewed product than earlier brewing steps. Future work focusing on archaeological examples will also be critical to understand diagenesis, but this marks a first step towards identifying protein-based indicators for brewing activity in archaeological contexts. Additional experiments aiming to investigate a) conditions of beerstone formation and b) variation in the protein composition of beerstone formed from different brewing recipes will be critical in understanding the viability of this substrate in future applications.

CRediT authorship contribution statement

Lindsey Paskulin: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Krista McGrath:** Writing – review & editing, Methodology. **Richard Hagan:** Writing – review & editing, Formal analysis. **Camilla Speller:** Writing – review & editing. **Marian Berihuete-Azorín:** Writing – review & editing, Resources. **Hans-Peter Stika:** Writing – review & editing, Resources. **Soultana-Maria Valamoti:** Writing – review & editing, Resources. **Jessica Hendy:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2024.106019.

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