

Article

Revealing the 2300-Year-Old Fermented Beverage in a Bronze Bottle from Shaanxi, China

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Abstract: China has a 9000-year-long history of cereal-based alcohol production, with the use of molds (filamentous fungi) likely being one of the earliest fermentation techniques. This method later developed into the uniquely East Asian *qu* (koji) starter compound, containing grains, molds, yeasts, and bacteria. Recent studies have revealed that this method was already widely applied during the Neolithic period. However, much less is known about its development during the early dynastic times, and our knowledge of this innovation has mainly relied on textual materials. Here, we present direct evidence, based on microbotanical, microbial, and chemical analyses, for the fermentation method of a 2300-year-old liquid preserved in a sealed bronze bottle unearthed in a Qin tomb at Yancun, Shaanxi. The results of this research suggest that this liquid is likely a fermented beverage made from wheat/barley, rice, Job's tears, broomcorn millet, and pulses. The fermentation starter may have been a cereal-based *qu*, consisting of a wide range of microorganisms, including molds (*Aspergillus* and *Monascus*), yeasts, and bacteria. Our findings suggest that the tradition of selecting suitable grains and microbial communities for brewing alcohol, possibly with a *mai**qu* starter (primarily wheat/barley-based *qu*), may have been well established more than two thousand years ago.

Keywords: Qin tomb; starch granules; yeasts; filamentous fungi; *qu* starter; wheat/barley; rice



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1. Introduction

China boasts a rich history of cereal-based alcohol production. Recent years have witnessed significant advancements in our understanding of the origins of alcohol production in China, thanks to the application of microanalytical methods in archaeology. Through chemical, microbotanical, and microbial analyses of residues adhering to and absorbed in pottery vessels from over 20 Neolithic sites (ca. 9000–3800 cal. BP) in the Yellow River and Yangzi River regions, direct evidence of alcohol production has emerged [1]. The findings indicate that fermented beverages were crafted from a variety of grains, predominantly millets and rice, alongside Job's tears and pulses. Tubers such as yam, lily, snake gourd root, and ginger were also utilized [2–5], sometimes accompanied by honey and fruits [6,7]. These alcoholic beverages were not only served to the living but also offered to ancestors as part of grave goods [8].

Among these discoveries, while some indicate the utilization of the malting method for saccharification [3], the majority indicate that the brewing technique corresponds with

the *qu* (or *koji*) fermentation process [4,5]. The latter method involves using moldy cereals to create the *qu* starter compound, comprising a range of filamentous fungi (such as *Aspergillus*, *Rhizopus*, *Mucor*, and *Monascus* molds), yeast, and bacteria. This *qu* mixture is then combined with steamed or boiled starchy-rich grains for fermentation [9,10]. Furthermore, specific types of plants may be added to the *qu* starter, known as *caoqu* (herbal *qu*), since these plants are likely abundant in microorganisms such as yeast, which can enhance fermentation efficacy [11,12]. In this method, filamentous fungi are the core components of the *qu* starter, as they secrete various enzymes to degrade starch material into fermentable sugars, while yeast converts sugars to carbon dioxide and alcohol. Together, they help achieve saccharification and fermentation simultaneously.

The Bronze Age in China, beginning around 1900 BC, was characterized by bronze vessels made for ritual feasting and sacrifice, often involving fermented alcoholic beverages. This tradition continued for millennia during the dynastic period. Occasionally, well-sealed bronze vessels containing liquid have been found during archaeological excavations, providing valuable opportunities for scientific analyses to investigate the nature of the contents. At least 13 such vessels, dating from the late Shang to the Warring States period (ca. 1100–221 BC), have been unearthed from tombs in North China. Of these, only six have been subjected to scientific analyses. These analyses, though differing in methodology and quality, offer substantial insights into the contents of the vessels, predominantly fermented beverages. However, these studies do not consistently reveal the fermentation methods employed (see SI, Table S1 for details and references).

It is intriguing that we possess far less knowledge about fermentation techniques pertaining to the well-preserved liquid in bronze vessels compared with the residues found in Neolithic pottery sherds. This irony deepens considering that various fermentation methods had already been documented in the earliest written records from the Bronze Age. In the oracle-bone inscriptions of the late Shang dynasty (ca. 1250–1046 BC), three types of alcoholic beverages were mentioned: *li*, *jiu*, and *chang* [13]. The Zhou Dynasty document, *Shangshu* (*Book of Documents*; possibly compiled in the fifth century BC), further describes that *li* alcohol was crafted from *nie* (malted cereals), *jiu* from *qu* (moldy grains), and *chang* from a blend of millet and herbs [12,14]. By the Eastern Zhou period (770–221 BC), wheat and/or barley were predominantly used as primary ingredients for producing *qu*, known as *mai**qu* (wheat *qu*) [15].

Several ancient documents from historical periods provide additional information about the methods for making *qu* and brewing fermented beverages. For example, *Qimin Yaoshu* (*Essential Techniques for the Welfare of the People*), dating to 533–544 AD, discusses more than ten methods for making *qu* starters. Additionally, *Tiangong Kaiwu* (*The Exploitation of the Works of Nature*), published in 1637 AD, details the process of making the red rice *qu* starter [16]. However, despite the detailed written records about brewing methods, we have little archaeological evidence of fermentation techniques from historical periods to verify ancient texts. This issue is partially related to the analytical methods employed, which are primarily chemical testing.

Different analytical methods have their own advantages and drawbacks. Chemical analyses, often requiring sophisticated equipment, are effective for identifying various biomarkers in the organic remains of fermented beverages, including beer and wine, in brewing vessels [17,18]. However, they are not sufficient for distinguishing different brewing techniques traditionally used in China [7]. Analyses of microfossil remains, including microbotanical (starches and phytoliths) and microbial (molds and yeasts) ones, require less-expensive equipment while providing reasonably precise identification of plant origins (grains and tubers) in the fermentation ingredients, as well as revealing different brewing methods (the use of malts and *qu* starters). These approaches are effective for testing cereal-based alcoholic beverages [2,3].

Therefore, to provide a more holistic picture of alcohol production, it is ideal to test ancient samples with a combination of chemical and microfossil analyses, as exemplified by a recent study of the liquid in a Han Dynasty (202 BC–AD 220) bronze vessel [19]. Here, we

present the study of a 2300-year-old liquid in a bronze *hu* bottle excavated from a tomb at the Yancun cemetery in Shaanxi province by integrating both types of analyses. This project was a collaborative effort between multiple institutions, where researchers employed various methods to study the nature of this rarely preserved liquid, as described below.

2. Archaeological Materials

The Yancun cemetery in Xianyang city, dating from the late Warring States period to the Qin dynasty (ca. 300–206 BC), is situated on high ground (453 m in altitude) north of the Wei River, which is part of the Xianyang Plateau region (Figure 1A). This plateau is formed by loess deposits 80–100 m thick, measuring 32 km E-W and 10–13 km N-S, with an altitude of 420–510 m, 70–100 m higher than the floodplains of the Wei River to the south. The water table is currently 30–50 m below ground level. Such a geographical configuration (high ground and dry conditions) has long been preferred for burials throughout history; thus, numerous ancient tombs, including dozens of imperial mausoleums from many dynasties, have been discovered there [20,21].



Figure 1. Site locations and artifacts from tomb M41 discussed in this paper. (A) Locations of sites revealing alcohol remains. 1: Yancun; 2: Beibai'e; 3: Anyang (Liujiashuang and Dasikong); 4: Qianzhangda; 5: Changzikou; and 6: Tianhu. (B) Tomb M41, showing grave goods in the left chamber; (C) the interior of the lid from the *hu* bottle, showing corroded surface and remaining textile used to help seal the vessel; (D) tightly lidded bronze *hu* bottle; (E) M41 plan; (F) liquid sample analyzed in this study; and (G) the *hu* bottle (middle) with other vessels excavated in the tomb (photographs of the site and artifacts were taken by Yanglizheng Zhang).

The Yancun cemetery comprises more than 100 tombs belonging to commoners of the Qin ethnic group. Among these tombs, M41 is a double-chambered burial, measuring 3 m in length at the bottom and 5.6 m in depth. It is located well above the water table, and the excavation revealed no signs of waterlogging. The tomb was furnished with nine grave goods, including one bronze mirror and eight pottery and bronze vessels, among which was a tightly sealed bronze *hu* bottle (30.4 cm in height and 21.6 cm in maximum body diameter) [22,23].

When unearthed, the bottle was tilted, and its mouth was covered with a textile made of bast fibers tied with a strip of plant stalk, then sealed with a bronze lid. Upon removing the lid, about 300 mL of liquid was found inside the vessel. The liquid was first carefully transferred to a clean glass jar, then sealed in a laboratory container, and stored in a refrigerator in the Shaanxi Institute of Archaeology in Xi'an. A small sample of the liquid was taken for analysis before being sealed for storage, with some visible impurities noted at the bottom of the glass bottle (Figures 1 and S1). The liquid was odorless and transparent, with a metallic flavor according to two people who tasted it.

The *hu* bottle, formed with a narrow neck and large belly and covered with a lid, is commonly regarded as an alcohol storage vessel. This vessel type, made of bronze or pottery, has frequently been found as part of grave-good assemblages in Qin burials during the Eastern Zhou period [24]. This discovery, therefore, provided a rare opportunity to study the fermentation method and alcohol-related mortuary practices in early China.

3. Research Methods

We utilized various methods to investigate the nature of the liquid, postulated to be the remains of a fermented beverage. Our research methodologies included tests for fatty acids and amino acids, analyses of the morphological characteristics of microfossil remains (both microbotanical and microbial) using light microscopy, and identification of chemical biomarkers via mass spectrometry (MS) analyses. Additionally, we conducted comparisons between the Qin liquid and five control samples obtained from sediments and well water near tomb M41. These control experiments involved testing for microorganisms and biomarkers as well as measuring pH values.

3.1. Fatty Acid and Amino Acid Tests

We applied 200 μ L of 2.5 mol/L ammonia to approximately 5 mL of the Qin liquid sample. The mixture was then centrifuged at 3000 rpm for 2 min. The upper layer was removed and transferred to a new clean tube and then distilled to dryness and dissolved in 100 μ L of trifluoroacetic acid. Free organic acids were extracted with ethyl ether and mixed with the solid residue from the ammonia extraction. The mixture was saponified with 200 μ L of a 10% (weight) KOH-ethanol solution assisted by a microwave. After saponification, the alcoholic solution was extracted with 200 μ L of hexane, and the remaining solution was acidified with 6 M HCl and then extracted again with 200 μ L of ethyl ether. The extracts from the hexane and ethyl ether were combined with tridecanoic acid (as an internal standard), dried under nitrogen gas, and derivatized with 20 μ L of BSTFA and 50 μ L of iso-octane for fatty acid testing.

The lower layer after ethyl ether extraction was used for protein purification with Omix C4 SPE cartridges. The protein was eluted with a mixture of 0.1% methanoic acid, 75% methanol, and 25% water, dried under nitrogen gas, and then hydrolyzed with 6 M HCl, assisted by a microwave. The solution after hydrolysis was applied to norleucine (as an internal standard), dried under nitrogen gas, and derivatized with 10 μ L of MTBSTFA (with 1% TMCS).

Two microliters of the derivatized solution was used for amino acid testing using a 7890A-5975C GC-MS (produced by Agilent Corporation, Santa Clara, CA, USA). We used an amino acid standard solution containing 12.5 μ mol/mL of proline and hydroxyproline, as well as 2.5 μ mol/mL of aspartic acid, glutamic acid, alanine, arginine, cysteine, phenylalanine, glycine, hydroxylysine, isoleucine, histidine, leucine, lysine, methionine,

serine, tyrosine, threonine, and valine. The fatty acids and dicarboxylic acid acetone solution included lauric acid (0.24 mg/g), succinic acid (0.27 mg/g), azelaic acid (0.28 mg/g), myristic acid (0.25 mg/g), succinic acid (0.3 mg/g), palmitic acid (0.25 mg/g), oleic acid (0.51 mg/g), and stearic acid (0.51 mg/g). The internal standard leucine aqueous solution (purity 99%; 138.66 µg/g) for the amino acid analysis and the internal standard isooctane solution (purity 99%, 135.48 µg/g) for the fatty acid analysis were purchased from Sigma Corporation in the United States.

For example, in the amino acid analysis, standard solutions with different concentration gradients were measured. The working curve was plotted with the amino acid content (µg/mL) as the *x*-axis and the peak area as the *y*-axis. The linear correlation coefficients (R^2) of the 11 amino acids ranged from 0.9916 to 0.9989. The detection limit of the protein was calculated as the sum of the detection limits of the 11 amino acids, totaling 0.31 µg/g. Similarly, the linear correlation coefficient (R^2) for the 8 fatty acids and dicarboxylic acids ranged from 0.9951 to 0.9999. The detection limit of the fatty acid analysis method was calculated as the sum of the detection limits of the 8 fatty acids and dicarboxylic acids, totaling 3.46 µg/g.

This part of the project was conducted at Northwest University in Xi'an.

3.2. Analyses of the Microfossil Remains

Microfossils often refer to plant micro-remains such as phytoliths, pollen, and starch grains recovered from archaeological contexts [25]. We extend this concept to describe microbials, specifically fungi and bacteria, which have also been found in ancient remains, particularly in the remnants of fermented beverages. In recent years, we have carried out a series of fermentation experiments using various plants and brewing methods. Additionally, we have conducted ethnoarchaeological investigations to understand fermentation processes, including the use of malts and the preparation of *qu* starters for brewing beer [26,27]. We collected samples of *qu* fermentation starters, yeasts, bacteria, and alcohol products from these producers and also tested a traditional homemade millet beer from Shimao, north Shaanxi, using DNA sequencing ([2,28]; SI Appendix). The samples and data from these studies, which formed part of the modern comparative collection in the Stanford Archaeology Centre, were used as references in the current project. The identifications of the ancient microfossils were based on these modern references, as well as on consultation with published information for starch [26,29] and fungi [10,30]. Accordingly, the utilization of the *qu* method was deduced when specific filamentous fungi, yeast cells, and starch granules displaying signs of fermentation-induced damage were found in significant concentrations within the examined vessels [1].

For the current study, a sample of the Qin liquid was obtained for microfossil analysis (Figure 1F). A small amount of liquid (ca. 30 µL) was extracted with a pipette from the bottom of the sample, transferred onto a glass slide, covered with a coverslip, and sealed with nail polish. Five slides were made and observed under a Zeiss Axio Scope A.1 (400× and 1000×, Carl Zeiss AG, Jena, Germany). Images were taken using a Zeiss AxioCam HRC3 digital camera and Zeiss AxioVersion software Version 4.9.1. This part of the project was carried out at Stanford University.

3.3. Identification of Microfossils Based on DNA-Sequenced Modern Samples

We attempted but failed to recover ancient DNA in the Qin liquid. Therefore, in order to identify the microorganisms in the Qin liquid sample, we analyzed the morphological characteristics of yeasts, bacteria, molds, and cereal grains detected in the DNA sequence of a traditional homemade millet beer from Shimao, also in Shaanxi. This unfiltered, low-alcoholic drink is made of sprouted and ground wheat or maize as a fermentation agent, mixed with steamed broomcorn millet flour, and fermented by wild yeasts in the environment ([2,28], SI Appendix). The composition of microorganisms in this beverage was used for comparison with that in the ancient liquid.

3.4. The MS Analyses

Mass spectrometry analyses were conducted using a Fourier-transform ion cyclotron resonance mass spectrometer (FTICR MS, Bruker Daltonics Inc., Bremen, Germany) equipped with a 7.0 T actively shielded superconducting magnet (MagneX, Oxford, UK) and an electrospray ionization source (ESI, Apollo II, Bruker Daltonics, Bremen, Germany). The ESI conditions included a drying gas temperature of 200 °C, and a nebulizing gas flow rate of 1.0 L/min, with 4.4 kV applied on the atmospheric side of the glass capillary and 3.9 kV on the atmospheric chamber end cap shield. The capillary exit voltage was maintained at 36 V to prevent capillary–skimmer dissociation at the ESI interface. Ion excitations were conducted in broadband mode using a frequency sweep radial ion excitation technique.

In this study, a tune mix standard solution was used for calibration. To achieve a mass accuracy error of less than 2 ppm, the commercial ESI tune mix was diluted with acetonitrile at a ratio of 1:100. The detection limit of the analysis depended largely on the ionization efficiency of the molecules under examination, typically reaching levels as low as parts per billion (ppb). Due to the small sample quantities and limitations, a direct injection method was employed for detection, with intensity serving as a key reference point for quantification.

The samples underwent the following processing steps: (1) For the Qin liquid sample and modern well water, 200 µL of the liquid was centrifuged at 2000 rpm for 5 min, and the supernatant was transferred to a new clean 1.5 mL tube. (2) For each of the four sediment samples, 1 gram of sediment was weighed into a 5 mL EP tube, and 2 mL of deionized water (DI) was added. The mixture was vortexed for 30 s and centrifuged at 2000 rpm for 5 min, and approximately 200 µL of the supernatant was transferred to a new clean 1.5 mL tube. (3) All the above solution samples were diluted with DI water and then injected directly into the ionization source via a microliter pump at a flow rate of 120 µL/h.

This part of the project was conducted at Xiamen University.

3.5. Microfossil Analysis of Control Samples

Since tomb M41 became inaccessible after excavation, control sediment samples were collected from a construction ditch located 20 m away from the tomb. These samples were obtained at depths of 1, 2, 3, and 4 m below ground level, labeled YM01-04. Approximately 5 g of each sample was collected using a clean trowel and sealed in a clean plastic bag.

The control samples were processed according to the following procedure: 1 gram of each sediment sample was weighed and placed into a 5 mL EP tube. Then, 2 mL of sodium polytungstate heavy liquid (density 2.0) was added and mixed with a vortex mixer for 30 s. The sediment–heavy liquid solution was centrifuged at 2000 rpm for 5 min. Approximately 100–200 µL of the light fraction was transferred to a new, clean 1.5 mL tube, and deionized water was added to reach a final volume of 1.5 mL. The solution was vortexed and centrifuged again at 5000 rpm for 5 min, after which the supernatant was decanted. This step was repeated three times to ensure the complete removal of the sodium polytungstate. Approximately 20–50 µL of the solution containing the residues remained at the bottom. This solution was mounted on a glass slide with 50% glycerol, covered with a coverslip, and sealed with nail polish.

A water sample from a local well located approximately 20 m from the tomb was also collected as a control, using a clean 10 mL EP tube. An amount of 1 milliliter of the water sample was transferred to a new, clean 1.5 mL EP tube and centrifuged at 5000 rpm for 5 min. After centrifugation, the supernatant was decanted, leaving approximately 50 µL of water at the bottom. The slide was then prepared in the same manner as the sediment samples.

All slides containing control samples were observed using a Zeiss Scope A1 microscope (Carl Zeiss AG, Jena, Germany) equipped with a Zeiss Axiocam MRC camera and recorded using the Axiovision software. This test was conducted at Xiamen University.

3.6. Testing pH Values

We tested the pH values of both the Qin liquid and the control samples. The tests were conducted at 20 °C using pH indicator papers from Geshan Company, China, with an accuracy of 0.5. For the Qin liquid and the well water, approximately 50 µL of each sample was applied onto the indicator papers to observe any color change. For the sediment samples, approximately 1 g of the sediment was mixed with deionized water in a 1:1 mass ratio, following the method outlined by Smith and McGrath [31]. The resulting slurry was thoroughly mixed by ultrasonic shaking and then allowed to stand for 30 min. The supernatant was subsequently used for pH testing.

The pH tests were conducted at Xiamen University.

4. Results

4.1. Fatty Acid and Amino Acid Testing

In order to determine whether the liquid content originated from underground water or the remains of artificially created matter, gas chromatography–mass spectrometry was employed to analyze the amino acids and fatty acids extracted from the liquid. The results show that the content of common amino acids was higher than the detection limit of the method (referring to the minimum concentration or content of an element required to produce an analytical signal that can be reliably detected), suggesting that the sample contained detectable free amino acids. Among these, hydroxyproline and glutamic acid occurred in relatively high concentrations (4.83 µg/g and 2.40 µg/g, respectively). A small quantity of fatty acids was also detected in the sample, mainly diacid acid and lauric acid (1.37 µg/g and 0.62 µg/g, respectively). The preliminary results of the tests were published in the excavation report [22] (see Figure S2).

4.2. Microfossil Analyses

Abundant microfossil remains ($n = 1522$ particles in total) in the Qin liquid sample were recorded, including starch granules, yeast cells, bacteria, and molds (Tables S2 and S3). Most particles appeared yellow-greenish or reddish in color, probably affected by metallic particles while stored in the bronze vessel, which is apparently corroded (Figure 1C), for two thousand years. Several bast fibers were present, likely derived from the textile that covered the vessel mouth. To test the origins of these microfossil remains in the liquid, we analyzed control samples from local sediments at different depths and underground water.

4.2.1. Starch Granules

A total of 322 starch granules were recorded. Among them, 299 (92.9%) could be classified into four types identifiable to certain taxa. Almost all granules appeared damaged, showing blurry extinction crosses, broken edges, deep channels or fissures, pitting on the surface, a depression in the center, or gelatinization (Figure 2). These features are consistent with those affected by enzymatic digestion and cooking, commonly found in fermented and cooked foods in experimental studies (Figure S3) [26,27,29]. The taxa of 23 granules (13.5%) were unidentifiable (UNID) due to a lack of diagnostic features or severe damage (Figure 2I,J,M; Table S3).

The type I granules ($n = 19$; 5.9% of the total) were slightly polygonal in shape, measuring 7.06–27.04 µm in maximum length. The hila were centric, and the extinction crosses were “+” shaped. These characteristics resembled those of the Panicoideae subfamily, including broomcorn millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), and Job’s tears (*Coix lacryma-jobi*), all domesticated in China [32]. One relatively large granule with a zig-zag-shaped arm was typically consistent with Job’s tears (Figure 2A,B compared with Figure S3A,B), based on previous studies [33]. When the starch morphology and size ranges were compared, type I fell into the combined size range of broomcorn millet, foxtail millet, and two variants of domesticated Job’s tears (*C. lacryma-jobi* var. *ma-yuen* and *C. lacryma-jobi* var. *lacryma-jobi*) (Figure S4A).

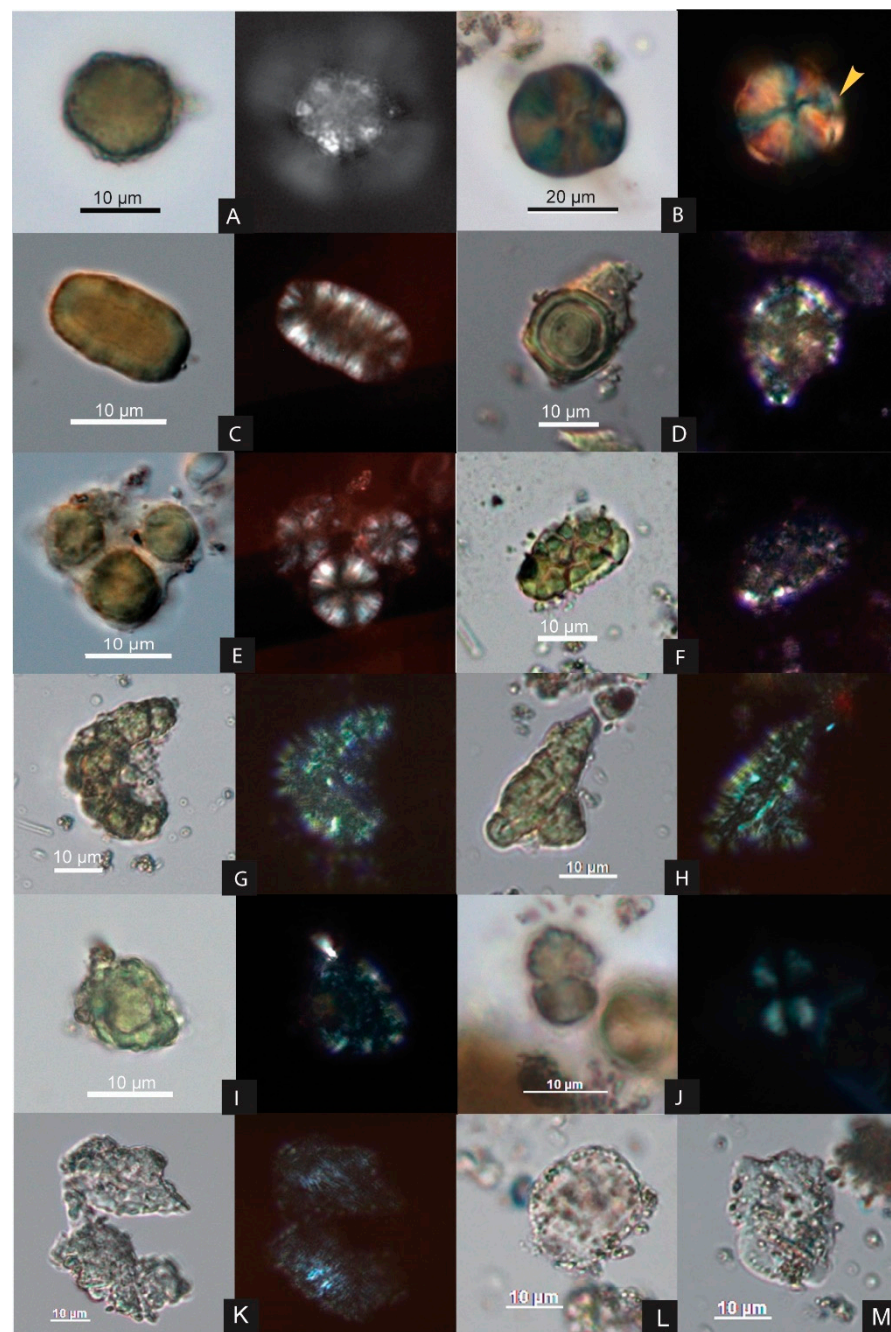


Figure 2. Starch types from the Qin liquid. (A) Type I, Panicoidae, likely millet; (B) type I, Panicoidae, likely Job's tears (arrow pointing to the zig-zag arm; (C) type II, pulses; (D) type III, Triticeae (A-type), showing deep channels; (E) type III, Triticeae (B-type), showing deep channels, a central depression, and pitting; (F) type IV, rice compound; (G) type IV, rice compound, partially missing; (H) mildly gelatinized starch masses, possibly rice; (I) UNID starch, showing hollowed center; (J) UNID starch, broken edges; (K) gelatinized starch mass, possibly rice; (L) gelatinized starch, resembling wheat/barley; and (M) UNID gelatinized starch. (A–K) Each image shown in bright field and polarized views).

The type II granules ($n = 10$; 3.1% of the total) were comparable to Fabaceae pulses, possibly wild pea *Vicia* sp. or domesticated pea *Pisum sativum*. They were elongated–oval, measuring 13.19–24.85 μm in length, the hila were centric, and the extinction crosses were blurred, forming a dark, elongated area in the center with multiple fissures. The central depression and broken body were also similar to the damage on the fermented pea

starch in our modern reference (Figure 2C compared with Figure S3C). *Vicia* wild pea is native to China; there are 17 species of *Vicia* growing in Shaanxi, one of which, *V. gigantea* Bge [34], was referred to as *wei* in ancient texts and used for food in the Zhou dynasty [35]. Domesticated pea (*P. sativum*), a Near Eastern crop, was introduced to China probably around the Eastern Zhou dynasty, as inferred from the presence of a charred seed at the Dongyang site in Huaxian, Shaanxi [36]. Starch granules from wild and domesticated peas are morphologically similar; thus, it is not possible to make further taxonomical identifications of type II starch.

The type III granules ($n = 242$; 75.2%) were consistent with Triticeae, with a size range of 4.36–33.33 μm . The hila were centric, and the extinction crosses were “+”-shaped. Triticeae starch granules exhibit a bimodal size distribution, consisting of large granules, lenticular in 3D (A-type; $>10 \mu\text{m}$), and small, almost spherical, granules (B-type; $<10 \mu\text{m}$). The type III granules exhibited various damaged features, such as pitting, central depressions, concentric channels, and gelatinization, resembling those found in fermented wheat (*Triticum* sp.) and barley (*Hordeum* sp.) starch in our brewing experiments (Figure 2D,E,L compared with Figure S3D,K,L). Wheat and barley were introduced to China by the second millennium BC and gradually became widespread thereafter [37].

The type IV granules resembled rice (*Oryza* sp.). They were small, appearing as compound starch clusters. The granules were polygonal in shape, and the extinction crosses were unclear. These characteristics were consistent with fermented rice starch in our modern database (Figure 2F,G compared with Figure S3E,F). We recorded 28 rice compounds (8.7% of the total) and measured 51 granules (size 2.54–6.56 μm). Several gelatinized starch masses also exhibited a granulated pattern, resembling fermented and cooked rice compounds (Figure 2H,K compared with Figure S3H,I). Rice has been cultivated in China since Neolithic times, but it was rare during the Zhou period (the 1st millennium BC) in the Wei River region where the Qin tomb is located [38].

In summary, the damage patterns on the starch granules were consistent with fermentation, indicating the nature of the liquid as a fermented beverage. The fermentation ingredients may have included wheat/barley, rice, broomcorn millet, foxtail millet, Job’s tears, and pulses (likely peas). Wheat/barley accounted for 75% of the starch assemblage, indicating their prominent presence among the fermentation ingredients. These grain types, except for wheat/barley, have been frequently uncovered from residues in fermentation-related Neolithic pottery vessels [1].

In the *qu* fermentation method, the *daqu* compound (big *qu*, used for producing yellow beer and distilled liquor) is normally made of uncooked grains, while the fermentation ingredients are well-cooked [9,10]. Our brewing experiment demonstrated that starch granules from the *qu* compound were relatively well preserved in the beverage when the fermentation was finished, while the steamed or boiled ingredients were almost completely gelatinized and lost their original morphology [27] (Figure S3J). Therefore, the starches that survived in the Qin liquid likely represented the ingredients in the *qu* starter, which was a mixture of multiple grains, consisting of predominantly wheat/barley, as well as rice, millets, Job’s tears, and peas. In contrast, the gelatinized starch masses, some resembling rice and wheat/barley, were likely associated with the cooked ingredients used in fermentation.

4.2.2. Yeast Cells

The most numerous microparticles recorded are yeast cells ($n = 689$), which can be classified into three groups: (1) oval or round ($n = 616$; 2.9–10.83 μm in length); (2) elongated-oval ($n = 31$; 3.32–9.88 μm in length); and (3) irregular rod-shaped ($n = 42$; 3.06–14.71 μm in length). Many cells exhibit characteristics of a budding process, such as a small protuberance on the parent cell, a smaller cell attaching to the bigger parent cell, or two cells of similar size connecting to one another (Figure 3A–E; Table S3). Budding is a typical feature of many yeasts, as they reproduce asexually by the asymmetric division process [30]. In our brewing experiments, yeast cells became more dominant in the microbial assemblage

toward the end of the fermentation process [27], a scenario resembling that of the Qin liquid (Figure S3L).

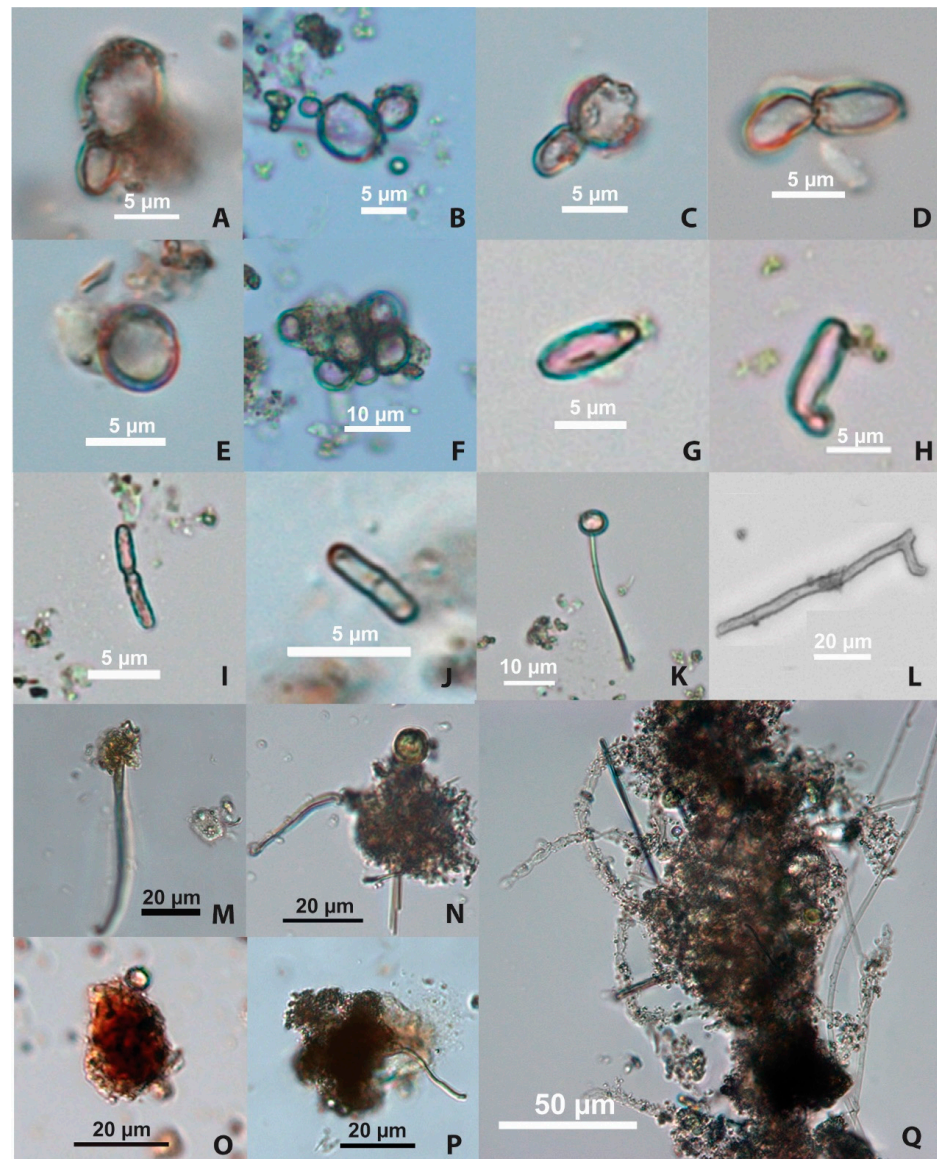


Figure 3. Fungi and bacteria in the Qin liquid. (A–D) Oval yeast cells in different stages of the budding process, cf. *S. cerevisiae*; (E) round yeast cell; (F) a cluster of oval yeasts; (G) elongated–oval yeast, cf. *Pichia* sp.; (H) rod-shaped yeast in budding; (I,J) rod-shaped bacteria in fission, cf. *Bacillum* sp.; (K) fungal spore in germination; (L) fungal hypha; (M) hypha associated with conidia; (N,P) conidial cluster connected with hyphae, resembling *A. niger*; (O) cleistothecium resembling *Monascus* sp., with a yeast cell on top; and (Q) mycelium attached to conidial mass, resembling *A. niger*.

In our DNA sequencing of a traditional home-made millet beer from Shimao, two of the most dominant yeast strains in the DNA sequence were *Saccharomyces cerevisiae* (3373 counts) and *Pichia kudriavzevii* (281 counts) ([2,28], SI Appendix), and cells morphologically similar to both yeast strains were microscopically identifiable. *S. cerevisiae* cells were round–ovoid/oval, and *P. kudriavzevii* (also named *Issatchenkia orientalis*, *Candida glycerinogenes*, and *Candida krusei*) were oval–elongated [39,40] (Figure 4A,B). We measured 131 yeast cells in the Shimao millet beer, including 125 *S. cerevisiae*, which were round ($n = 31$; diameter 3.47–6.63 μm) and oval/ovoid ($n = 94$; length 3.92–12.16 μm), and six *P. kudriavzevii* cells (length 11.78–16.47 μm). When comparing the Shimao wild yeast strains

with their cultured counterparts in our reference database (Figure 4C), we found the former were larger in size than the latter, probably reflecting the difference between wild and domesticated forms (Figures 3B and S4B; Table S4).

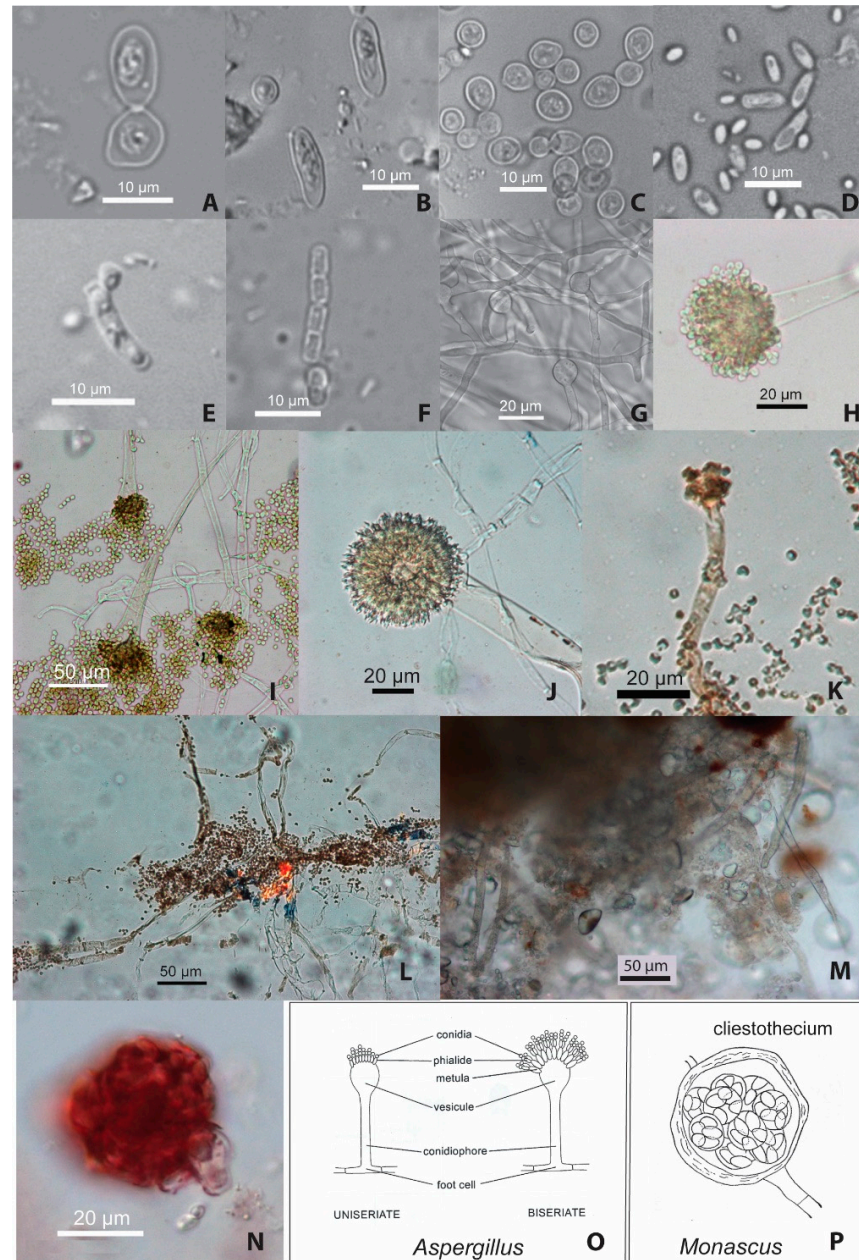


Figure 4. Modern yeasts and molds comparable to ancient fungal elements. (A) Oval and round yeast cells (wild *S. cerevisiae*) in Shimao millet beer; (B) elongated-oval yeast cells (wild *P. kudriavzevii*) in Shimao millet beer; (C) cultured *S. cerevisiae*; (D) cultured *P. kudriavzevii*; (E) rod-shaped yeast cell in *daqu*; (F) bacteria, cultured *Bacillus* sp.; (G) *Aspergillus oryzae* mycelium; (H) vesicle with conidia, *A. oryzae*; (I) mycelia and vesicles, *A. oryzae*; (J) vesicle with conidia, *A. niger*; (K) hypha with conidia, *A. niger*; (L) mycelia and conidia clusters, *A. niger*; (M) wheat starch and mycelium in millet beer, fermented for 12 days; (N) clialothecium, *Monascus* sp.; (O) structure of *Aspergillus*; and (P) structure of *Monascus* clialothecium.

Morphologically, the round and oval/ovoid cells in the Qin liquid were similar to *S. cerevisiae* (Figure 3A–F compared with Figure 4A,C), whereas the elongated-oval cells closely resembled *P. kudriavzevii* (Figure 3G,H compared with Figure 4B,D). When the sizes

were compared, the Qin round–oval and elongated–oval cells appeared smaller than the Shimao wild *S. cerevisiae* and *P. kudriavzevii*, respectively, but similar to their corresponding cultured cells (Figure S4B).

Several rod-shaped yeast cells show a budding process, similar to unidentified yeast in a *daqu* sample from a Chinese distiller (Figure 3H compared with Figure 4E). The Qin rod-shaped cells exhibited a wide size range, overlapping with both wild and cultured *P. kudriavzevii* as well as with the rod-shaped yeast from the *daqu* (Figure S4B). Apparently, the Qin yeast community consists of various species, not limited to *S. cerevisiae* and *P. kudriavzevii*, but some are currently unidentifiable.

S. cerevisiae, due to its high ability to ferment sugars to ethanol, is the most commonly used yeast for alcohol fermentation [41]. In Chinese *qu* starters, *S. cerevisiae*, *Pichia* sp., and many other genera (e.g., *Hanseniaspora*, *Hansenula*, and *Candida*) have been identified [9,42]. These yeasts contribute to the fermentation process in different ways. *S. cerevisiae*, for example, played a dominant role during the early stages of the fermentation process [43]. *P. kudriavzevii* produced about 35% and 200% more ethanol than *S. cerevisiae* at high temperatures of 40 and 45 °C, respectively [44], but also contributed to the negative effect of film-formation in fermented food [45].

4.2.3. Bacteria

Small rod-shaped cells ($n = 5$; length 4.59–8.79 μm) with features of binary fission in the cell division process were present in the Qin liquid sample. They most closely resembled rod-shaped bacteria, such as *Bacillus* (Figure 3I,J compared with Figure 4F) [46]. Diverse bacteria species form an important part of the microbial community in *daqu*, producing enzymes and contributing flavors to the alcohol products, and *Bacillus* strains have been found to be the most numerous [9,47,48]. Several *Bacillus* species have also been identified in the DNA sequence of the Shimao millet beer [28].

4.2.4. Filamentous Fungi (Molds)

Numerous fungal elements were recorded ($n = 536$), including hyphae ($n = 20$), mycelia ($n = 9$), fungal spores in germination ($n = 3$), conidial clusters resembling *Aspergillus* ($n = 472$), and cleistothecia likely from *Monascus* ($n = 2$) (Table S3). The hyphae and mycelia appeared either transparent or brown in color and were mostly septate (Figure 3L–N,P,Q). Some small spores were seemingly in a state of germination (Figure 3K). Many small, dark-colored spore clusters, in some cases connecting to hyphae or mycelia, were present (Figure 3M,N,P,Q). These mold particles resembled those of *Aspergillus*, particularly *A. niger* and *A. oryzae* (Figure 4G–L). These two species were very similar in our reference data, but the former produced smaller and darker conidia than the latter (Figure 4G–L,N). In the Qin liquid, the spores in those dark-colored spore clusters measured 0.96–2.79 μm , resembling those in *A. niger*, while two larger conidia during germination were transparent and measured 6.14 μm and 6.74 μm , falling within the range of *A. oryzae* (Table S4). Additionally, two cleistothecia were present, appearing red in color and containing numerous oval ascospores, which are typical characteristics of *Monascus* (Figure 3O, compared with Figure 4N,P). In our experimental brewing of millet beer, mycelia associated with starches were present in the beverage after the fermentation process was completed, a situation resembling that of the Qin liquid (Figure 4M) [27].

In China today, the dominant fungal strains in *daqu* include predominantly *Aspergillus*, *Mucor*, and *Rhizopus* [9,47,48], while *Monascus* is typically used to prepare rice-based *qu* starter for brewing red rice beer in southern China [49]. In the Qin liquid, the dominant fungal species appears to be *Aspergillus*, consistent with the *daqu* method.

4.3. MS Analyses

Previous studies have identified broomcorn millet as a main ingredient in Neolithic beer from the Wei River region based on starch and phytolith types [2,3,50]. In the Qin liquid, a small number of Panicoideae starch granules, possibly including millets, were

identified, although no corresponding phytoliths were present. Miliacin, the most abundant pentacyclic triterpene methyl ether in *Panicum* genus tissues [51], has been widely used as a molecular indicator of ancient broomcorn millet use [51–54].

Our FTICR MS tests revealed several peaks in the spectrum, including a quasi-molecular ion peak $[M+Na]^+$ at $m/z = 463.391040$ (Figure 5). This peak aligned with the theoretical $[M+Na]^+$ mass value of miliacin (3β -nethoxyolean-18-ene, $C_{31}H_{52}O$), which is 463.391037 (according to Bruker's Isotope Pattern of Compass DataAnalysis 4.0 software). The closeness between these values was well within the allowable instrument error of 5 ppm, making it acceptable for structural elucidation studies of the molecules with molecular weights of <1000 Da [55]. Therefore, the signal from the Qin liquid confirmed the presence of a small amount of miliacin. In contrast, no miliacin peak was observed in the spectrum of control samples from the sediments and well water. Given the weak miliacin signal indicating *Panicum*'s presence in the sample, its concentration was likely quite low, consistent with the small number of millet-like starch granules detected.

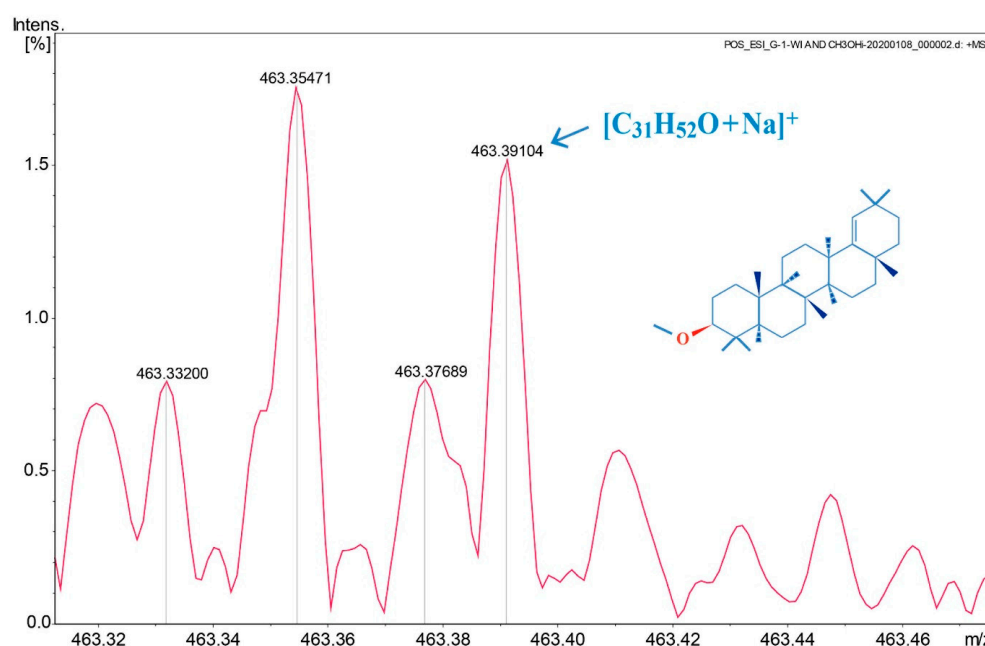


Figure 5. FTICR MS analysis showing the positive-ion mode of the Qin liquid sample, indicating the presence of miliacin.

4.4. Control Samples

The water sample contained very few fibers or plant tissues. All sediment samples contained pollens, charcoal pieces, fibers, and phytoliths, whereas only two samples (YM01 and YM04) revealed a total of six starch granules (one Triticeae and five unidentifiable). No yeast or other fungal elements were present. The control samples clearly differed from the Qin liquid sample in terms of their composition, confirming that the microfossil remains in the Qin liquid were not contaminated by the surrounding sediments or groundwater.

The pH values of the local sediment samples ranged from 7.0 to 7.5, indicating mild alkalinity. The well water's test result was 7.5, consistent with the sediments. The Qin liquid sample, however, had a pH value of 9.0–9.5, higher than all the control samples. The very high pH value of the Qin liquid may be attributed to its container, the corroded bronze vessel, as it is known that corroded bronze produces alkaline substances [56,57]. Considering the significant differences in pH between the Qin sample and local sediment-water samples, the content of the ancient liquid is unlikely to have been contaminated by the surrounding environment. Furthermore, pH testing has not been commonly applied to the research of ancient, fermented beverages, and our use of this method may draw attention in the academic community to the changes in pH values of fermented liquids during long-term burial processes.

5. Discussion

The presence of amino acids and fatty acids could serve as significant indicators of the organic remains in the vessel. However, as these chemical molecules are found in a wide range of organisms, they cannot be specifically attributed to a single organism or biological product. Additionally, heavy metal ions such as copper and lead, which are highly soluble in liquids within long-preserved bronzes due to the high pH value, may exhibit toxicity toward bacteria and other decay-causing organisms. This phenomenon could contribute to the preservation of organic matter [58,59], potentially explaining the high concentration of dead microorganisms and starch granules still present in the container.

The microfossil assemblage consisted of two major groups: starch granules and fungi–bacteria. While wheat/barley dominated the former, yeast and *Aspergillus* mold comprised the great majority of the latter (Figure 6). The starch granules with characteristics of fermentation, together with molds, yeasts, bacteria, and miliacin found in the Qin liquid, indicated the presence of a complex of starchy plants and microorganisms, attributable to a multi-grain fermented beverage using the *qu* method. The composition of microorganisms in the sediment control samples, as well as the pH value of the modern groundwater, markedly differed from those detected in the Qin liquid. This stark contrast further bolsters the results of the acid testing. Therefore, this suggests the ancient liquid sealed within the bronze bottle was very unlikely to be contaminated by the surrounding sediments.

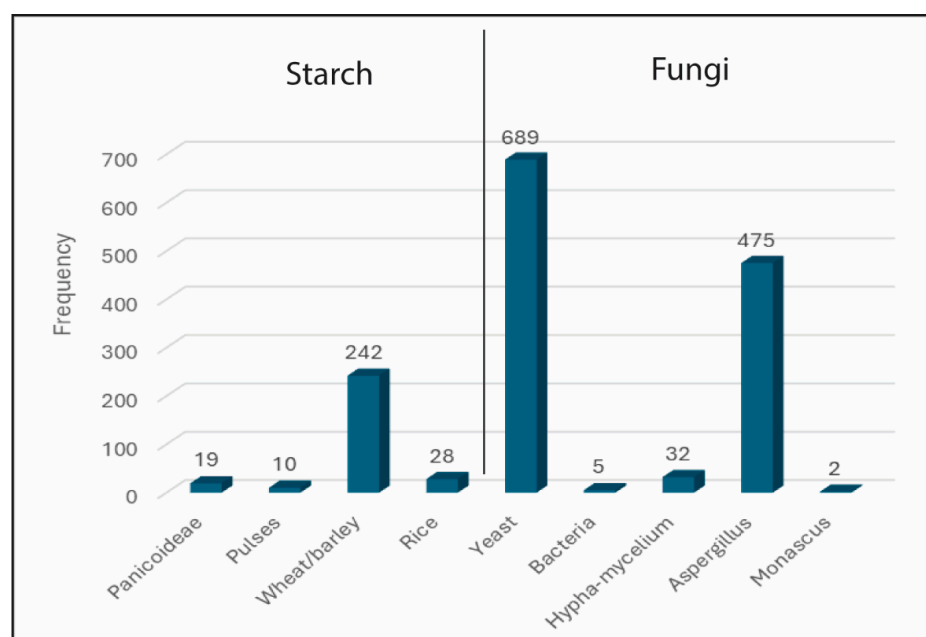


Figure 6. Comparison of major microfossil elements recovered from the Qin liquid.

The preserved starch granules were predominantly of Triticeae (wheat/barley; over 75%), supplemented by rice, Job’s tears, broomcorn millet, and pulses (possibly peas). These grains may have mainly come from the *qu* compound, which was likely uncooked, allowing the better survival of starch granules. This finding accords with the historical record of wheat *qu* (*mai-qu*) seen in the ancient texts, as aforementioned. The fermentation materials may have been cooked cereals, including rice and other grains, based on gelatinized starches, which could not be securely identified.

A. niger appears to have been the major saccharification agent in the *qu* starter, while other molds, *Monascus* sp. and possibly *A. oryzae*, may have also been part of the fungal community involved in the fermentation process. The fermented beverage was probably filtered but not distilled, as judged by its lack of solid sediments but the presence of abundant microfossils, a condition similar to the millet beer in our brewing experiment [27].

Most yeast cells resembled *S. cerevisiae*, with a few belonging to *P. kudriavzevii* and other species. Bacteria, such as *Bacillus*, may have also been functional microorganisms participating in the fermentation. Notably, the yeast strains of *S. cerevisiae* and *P. kudriavzevii* in the Qin liquid may be domesticated variants. Since China is likely the origin of domesticated *S. cerevisiae* [60], this finding will aid future studies on the evolutionary processes of amylolytic yeasts.

The grain ingredients in the Qin liquid, such as millet, Job's tears, rice, and pulses, were already used for alcohol fermentation during Neolithic times, but tubers and acorns found in Neolithic beer were lacking in the Qin sample. In contrast, the composition of the Qin *qu* ingredients was strikingly similar to those of *huangjiu* (yellow beer) and *baijiu* (white liquor) today. For instance, *huangjiu* is commonly made with wheat-based *daqu* starter and rice or millet as fermentation materials [61], whereas distilled liquors are made with *daqu*, in which wheat, barley, and pea are the core components, although sorghum is now the principal grain [9,62]. In either case, no tubers or herbs are added during the fermentation process. Given that the Qin bronze bottle was found in a commoner's tomb, it is possible that the use of wheat/barley-based *qu* for making *huangjiu*-like alcoholic beverages with rice and other cereals may have become a widespread practice by the Eastern Zhou period in North China.

Figure 7A presents a schematic process of *qu* compound production based on the findings of this study. A portrait brick from the Eastern Han dynasty (25–220 AD) in Sichuan depicts the process of fermentation, filtration, storage, and transportation of alcoholic beverages. This production scenario is likely comparable with the brewing method used in the Qin liquid studied here (Figure 7B). The brewing vessels, such as large vats and globular jars used for making *huangjiu* (Yellow beer) and *hongqujiu* (red rice beer) in Zhejiang today, were nearly identical to those depicted on the Eastern Han portrait brick (Figure 7C,D). These images collectively illustrate a continuous technical tradition of *qu* cereal-based fermented beverages in China.



Figure 7. Schematic process of brewing fermented beverages. (A) Making *qu* starter compound based on the findings of this study. (B) An Eastern Han portrait brick illustrating: 1. working on the brewing material containing *qu* and steamed cereals; 2. filtering fermented liquid through funnels into globular jars for storage; and 3. transporting alcoholic beverages by humans (artifact in Sichuan Museum). (C,D) Fermentation vat and storage jars from the Danxi Brewing Company in Zhejiang (photos by Li Liu).

6. Conclusions

This study demonstrates the advantage of an interdisciplinary approach to investigating ancient liquid alcoholic remains. By integrating multiple chemical analyses with microfossil studies, we could recover more information to reconstruct not only the components of the fermentation ingredients but also the brewing methods. The diversity and richness of the microorganisms matched well with the nature of *qu*-based alcoholic beverages, strongly excluding the possibility of non-fermentation liquid contents in the vessel. Analyzing control samples from sediments and groundwater at the site to compare with the liquid in the bronze vessel also helped to validate the authenticity of the ancient alcoholic liquid.

Our research suggested a diverse array of microorganisms in a 2300-year-old fermented alcoholic beverage crafted by a common Qin family as an offering to their deceased ancestors. The complexity of the microbial community within this beverage suggested a well-developed fermentation process, involving the preparation of a primarily wheat/barley-based, multi-grain *qu* starter for producing fermented beverages, which included rice, Job's tears, broomcorn millet, and pulses. This beverage bears the closest resemblance to *huangjiu*, or yellow beer, which is still widely produced today in China, indicating that this fermentation tradition was established over two millennia ago.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation10070365/s1>, Figure S1: Location of Yancun cemetery site; Figure S2: Selected ion chromatogram of fatty acids from liquid residues; Figure S3: Starch granules showing damages induced by fermentation in modern reference; Figure S4: Comparison of Qin Type I starch and yeasts with modern references. Table S1: Bronze vessels containing liquid unearthed in North China; Table S2: Counts and sizes of starch grains re-covered from the bronze bottle; Table S3: Counts and sizes (in μm) of fungi and bacteria recovered from the bronze bottle; Table S4: Measurements of Yeasts and conidia from Qin alcohol and modern reference [7,22,63–71].

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