Research article

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Preliminary microbiological and chemical analysis of two historical stock ales from Victorian and Edwardian brewing

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Historical beers are a valuable source of information on past brewing microbiology providing opportunity for analysis and isolation. Although rarely found intact and suffering variable degrees of deterioration they can contain living microbial cells and residues of chemical components indicating the character of the beers and their production processes. This report summarises preliminary analysis of two beers from Victorian and Edwardian times and provides an indication of their chemistry and microbiology. One beer, recovered from the 1895 Scottish shipwreck Wallachia, was a 7.5% ABV stout, the other an 11% ABV celebration King's Ale, a barley wine, brewed by Bass in 1902 for the visit of King Edward VII. Live yeast was isolated from both beers: *Brettanomyces* and *Debaryomyces* from the Wallachia stout and *Saccharomyces* from the Bass Ale. Ribosomal DNA amplicon sequencing indicated the presence of a wide range of microorganisms in both beers including lactobacilli and pediococci in the Wallachia beer and staphylococci in the Bass Ale. Both beers are likely to be in the category of stock ales of the time and would have been matured for a period before bottling. The presence of *Brettanomyces bruxellensis* in both beers confirms the understanding of this species as an important contributor to these beers but it is also interesting that *Debaryomyces* species are consistently present as a major component and that *Saccharomyces* are less prevalent. © 2021 The Institute of Brewing & Distilling

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Keywords: Historical beers; Wallachia; King's Ale; *Debaryomyces*; *Brettanomyces*; ribosomal DNA sequencing

Introduction

Bioarchaeology is a dynamic field of study with considerable relevance to the food industries in retrieving information on past foods and beverages *(1–6)*. Its methods have been applied to beer and extensive detail obtained regarding the ingredients and components of beverages from residues in prehistoric ceramics *(7, 8)* and in identifying evidence of early brewing *(9, 10)*.

A particularly interesting period of brewing history is between 1850 and 1950 during which scientific principles were applied and in which brewing microbiology was developed, initially by Pasteur *(11)* and subsequently by Hansen *(12)*, Jorgenson *(13)* and others. A pertinent issue in brewing microbiology around 1900 was the application of pure *Saccharomyces* yeast cultures developed by Hansen at the Carlsberg laboratory in 1888. These were readily adopted by continental breweries as providing more controlled production and purer beers *(14)*. Application to UK brewing was, however, less positively received, in part because of the belief that British beers possessed particular flavours arising from mixed yeast cultures and, specifically, the involvement of *Brettanomyces* species. This was especially believed to be essential for the character of 'stock' ales which were matured for extended periods *(15)*. While a number of breweries did try pure culture yeasts, UK brewing was resistant to change and, with the intervention of World War I, retained its indigenous yeast cultures *(16)*. Since the 1940's a more biotechnological approach to fermentation demonstrated the value of pure culture and was progressively applied to the larger breweries developing at that time.

During the formative period of brewery microbiology after Pasteur, brewing yeast were identified as *Saccharomyces* species based on morphological features of shape, filamentous propensity and spore characteristics *(17)*. Non brewing, 'wild' yeast was recognised and termed 'Torula' if non sporulating. Of these *Brettanomyces* strains were identified as contributing important character to stock ales *(15, 18)*. It is also clear from brewing texts *(19)* that bacteria were recognised as spoilage organisms in beer – as had been initially demonstrated by Pasteur in 1863. These species were mostly categorised as bacilli and typically portrayed as rods and associated with sarcina sickness – generally producing sourness. Some studies, nevertheless, identified lactic acid bacteria as indigenous components of standard beers *(20)*.

Today, wild yeast and bacterial contaminants of beer are extensively detailed *(21, 22)*. Wild yeast contaminants are associated with phenolic/medicinal flavours and bacteria with the production of lactic acid (lactic acid bacteria), acetic acid (acetic acid bacteria) and other off flavours including acetaldehyde, butyric acid, diacetyl, phenols and mercaptans *(23, 24)*. Considering the likely hygiene practiced in Victorian and Edwardian brewing it is probable that fermentations would be a mix of yeast species with possibly bacteria included. Illustrations of fermentations of the time indicate mixed cultures in forcing samples *(19)* while deposits

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collected in the 1950's and 1960's at the National Collection of Yeast Cultures (NCYC) are often a mix of yeast strains in any one brewery.

Contemporary breweries are increasingly interested in using novel microbiology, either unconventional yeast strains or mixes of species and strains for sour and natural products *(23, 25–28)*. Identifying the specific strains and species of yeast and bacteria present in Victorian and Edwardian beers is directly relevant to this and has particular value if cultures of authentic microorganisms can be retrieved. Reports of retrieved historic brewery microbiology are limited but hold interesting promise for identifying novel microorganisms. Recently the characteristics of *Lactobacillus* and *Pediococcus* strains isolated from a 170 year old shipwreck have been reported and related to contemporary strains *(29)*. Similarly, analysis of beer from an 1840s shipwreck investigated a range of yeast and beer flavours and interpreted these in the light of ingredients and yeast metabolism *(30)*. In the study reported here, samples of intact, historic beers were analysed for their character and microbiology to determine features relevant to their production and to assess potential for retrieving viable microorganisms.

Methods

Samples

Bottles of McEwan's stout were raised from the shipwreck vessel, Wallachia, in the Clyde estuary, 55 51 707 N, 004 57 189 W (GPS). This vessel sank after a collision in 1895 carrying a cargo of whisky, beer, acids, glassware and earthenware and lies in 30 meters of water as detailed at https://www.scottishshipwrecks.com/wallachia/. The corks of the bottles are inscribed with the brewer 'McEwan's', a major producer in Edinburgh since 1856. Three samples of the beer were received in September 2018 (W1), July 2019 (W2) and December 2019 (W3), and processed on receipt for chemical and microbiological analysis. Bottle W3 is shown in Figure 1A.

Bottles of King's Ale were purchased with unknown provenance but were intact with lead seals and full contents. The beer was brewed at the Bass brewery in Burton upon Trent in February 1902 to celebrate the visit to the brewery of King Edward VII and matured in cask until bottling in 1905, 1911, 1929 and 1977. It is not clear from the bottles which bottling run these originated. Bottles K1 and K2 contained full contents suggesting no shrinkage or leakage and are shown in Figure 1B with the intact seal of K1 shown in Figure 1C.

Figure 1. 1a. Wallachia bottle W3, 1b. King's Ale bottles, 1c K1 seal.

Yeast isolation from bottles and artefacts

Bottles were processed directly on receipt and uncorked with sterilised corkscrews in a biosafety category II recirculating cabinet using category II protocols in a location not used for processing brewery materials. Samples of beer were aseptically transferred into broth and onto agar media for growth analysis and incubated at 25°C for 14 days or until growth was evident. Growth was conducted in malt and nutrient broth and agar as non-specific media to grow fungi and bacteria respectively. Samples were also grown on more selective media: Wallerstein Nutrient agar for brewery microorganisms, lysine agar for non-brewing yeast and Raka-Ray agar for lactic acid bacteria. All cultures were incubated aerobically except Raka-Ray agar which was incubated anaerobically *(31, 32)*. Samples of isolated strains were deposited with the National Collection of Yeast Cultures, Norwich, UK under deposition numbers NCYC R798, NCYC R799 and NCYC R800.

Electron microscopy

Scanning electron microscopy samples were observed using a Hitachi S3000-N instrument with BSE and EDAX detectors. Applying an accelerating voltage of 20 kV at a vacuum pressure of 70 Pa allowed the samples to be viewed uncoated. Surface images and elemental composition data was collected via the SEM analysis and EDAX elemental profiling.

DNA extraction and purification

DNA was extracted from sediments and culture samples using Instagene® extraction matrix for fungal and Gram-positive and Gram-negative bacterial DNA according to the manufacturer's instructions (Bio-Rad). A pellet from a 1 mL beer sample or culture suspension was resuspended and incubated in 200 μL Instagene® extraction reagent for 30 minutes at 56°C with continuous agitation. Samples were then vortexed for 20 seconds followed by incubation at 100°C for 8 min. After centrifugation at 13,550 x g, supernatants were removed and stored at -20°C. DNA quantification was conducted on a NanoDrop Lite spectrophotometer (Thermo Scientific). As an internal control DNA from a mixed soil sample was extracted at the same time as isolations from W1 beer to determine the possibility of cross contamination. Of the 79 fungal species identified from BLAST matches in the soil sample none were common to the listing from the beer. (Supplementary information S1). All operations were conducted under containment conditions using safety category II protocols.

16S-rRNA gene and ITS sequencing and analysis

For identification of purified yeast colonies, the internal transcribed spacer (ITS) region of fungal rRNA cistron was amplified using primers ITS1 (5′-TCC GTA G GT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) *(33)*. Each PCR mixture contained 12.5 μL of Immomix® hot start polymerase enzyme (BioLine), 2.5 μL primer mixture (10 μM), 2 μL DNA and 8 μL PCR grade water.

Amplification was conducted in a BioRad T100 thermocycler. PCR protocol was an initial denaturation of 10 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds and extension at 72°C for 60 seconds. A final extension was at 72°C for 3 minutes. Sequences obtained have been deposited with GenBank with accession numbers MW158767-MW158769.

PCR products were visualised by electrophoresis on a 2% agarose gel in TAE buffer (pH 8.3) using GelRed dye (Sigma – Aldrich) and size measured against 50 bp ladder (Invitrogen). Bands were excised and DNA extracted using QIAquick gel extraction reagents (Qiagen) for Sanger sequencing conducted by Source Biosciences (Cambridge). Identification of Sanger sequences was conducted by BLASTn analysis against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the ISHAM barcoding database (http://its.mycologylab.org). Metagenomic profiling of extracted DNA was conducted by MRDNA services, Texas, USA (http://www.mrdnalab.com/sequencing-service.html) using Ion Torrent S5 SL PGM with primers 515F-806R for the V4 region of the bacterial 16S ribosomal RNA gene and ITS 1-4 for the fungal ribosomal cistron. The PCR primers were used in a single step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Ion Torrent PGM following the manufacturer's guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, sequences were depleted of barcodes and primers, then sequences <150 bp removed, sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp were also removed. Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a database derived from RDPII (http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov),

Accession number(s). Sequencing data were deposited at the Sequence Read Archive at NCIB under BioProject PRJNA671797 http://www.ncbi.nlm.nih.gov/bioproject/671797

Chemical analysis

All beer samples were clarified by filtration before analysis by standard EBC methodologies. Beer samples were analysed for alcohol by volume using an Anton Paar Alcolyzer Plus standardised against 5% v/v ethanol (Laboratory of the Government Chemist). Specific gravity was determined using an Anton Paar DMA 450 densitometer. Colour was determined by measuring absorbance at 430 nm. Bitterness was determined by extraction into iso-octane and measurement of absorbance at 275 nm. pH was determined using a Jenway 3510 pH meter and acidity by titration to pH 8.5 with 0.1 N sodium hydroxide. Chloride was determined using a Sherwood MKI Chloride Analyzer 926S according to the manufacturer's instructions.

Results

To answer the question of what information historic beers can provide, original samples from intact bottles were analysed for their chemical and microbiological characteristics.

Wallachia Ale: McEwan's Stout, 1895

Chemical analysis of basic beer characteristics of alcohol by volume, specific gravity, colour, pH and bitterness are reported in Table 1 for the three Wallachia samples. As the analysis of bottle W2 showed very different concentrations of the parameters, chloride levels were determined to test for possible dilution by seawater ingress. This indicated levels of 0.3 g/L for the beer in bottle W1 and 7.5 g/L for the beer in bottle W2.

Microscopy of sediment from bottle W1 are illustrated in Figure 2a and b, and indicated the presence of yeastlike cells (A), in some cases distorted (B), along with amorphous precipitates in the background of Figure 2. Larger, unidentified structures were also observed (C). Samples from W1 grew in malt and nutrient broths and produced hemispherical, white porcelain like colonies approximately 1.7 mm on both agar media after three days of growth (Figure 3a). Observation of the cells from these colonies showed spherical refractile yeast cells with visably limited internal contents (Figure 3b). The same microorganism was also isolated from broth incubated at a later time with samples of the cork. Scanning electron microscopy was conducted on the surface of the cork extracted from the bottle and indicated occasional clusters of yeastlike cells (Figure 3c). EDAX X ray analysis of the cork surface indicated a predominance of lead with occasional iron as the major ions detectable (Figure 3d). DNA extraction conducted from five independently isolated colony samples (four on malt agar and one on nutrient agar) amplified and sequenced with ITS primers indicated the yeast to be *Debaryomyces hansenii* with 99.8% identity over 574 nt aligned in BLASTn. Further molecular biology analysis was conducted on samples from the three Wallachia bottles using metagenomic profiling to identify the presence of past microorganisms. The range of fungi and bacteria with a greater than 1% proportion of reads from DNA isolated from bottle W1 is listed in Table 2. In total six species of fungi and 44 species of bacteria were identified. Full details of the bacteria species with proportion of reads <1% are reported in supplementary information Table S2. In addition to the four lactic acid bacteria with high proportions of reads, four additional lactic acid bacteria, *Lactobacillus camelia (reclassified as Lacticaseibacillus camelliae), Lactobacillus suebicus (reclassified as Paucilactobacillus suebicus), Pediococcus acidilactici* and another undefined *Pediococcus* were identified at lower proportions of reads.

Table 1. Chemical analysis of beer from Wallachia bottles.

Sample	ABV (%)	Original gravity	Present gravity	pH	Acidity % (v/v) as lactic acid	Colour EBC	Bitterness EBU
W1	7.5	1.0648	1.0075	3.63	0.576	299	18
W2	3.2	1.0421	1.0165	3.2	0.450	91	2.45
W3	7.5	1.0636	1.0068	3.28	0.630	209	25.20

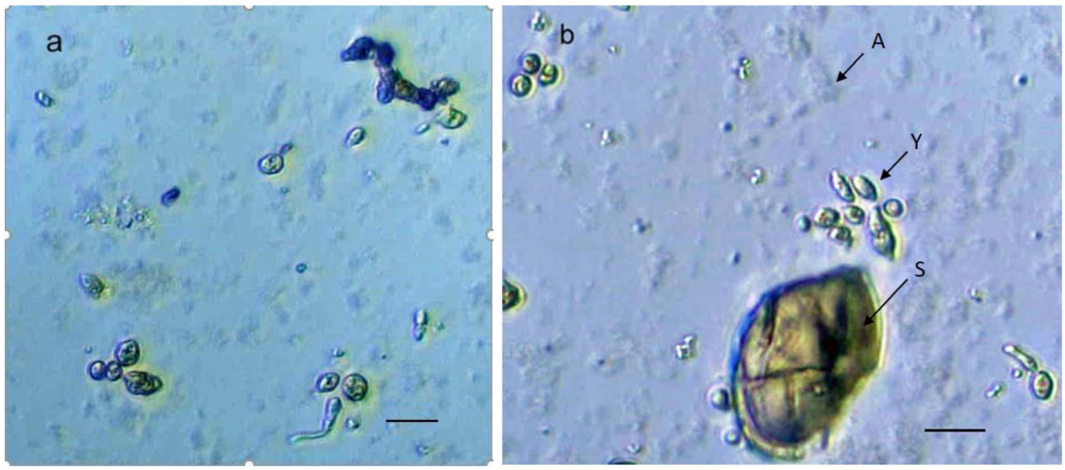

Figure 2. Sediment of beer isolated from bottle W1. A – Amorphous material: Y – yeast-like structures; S – unknown structure. Bar = 10 μm.

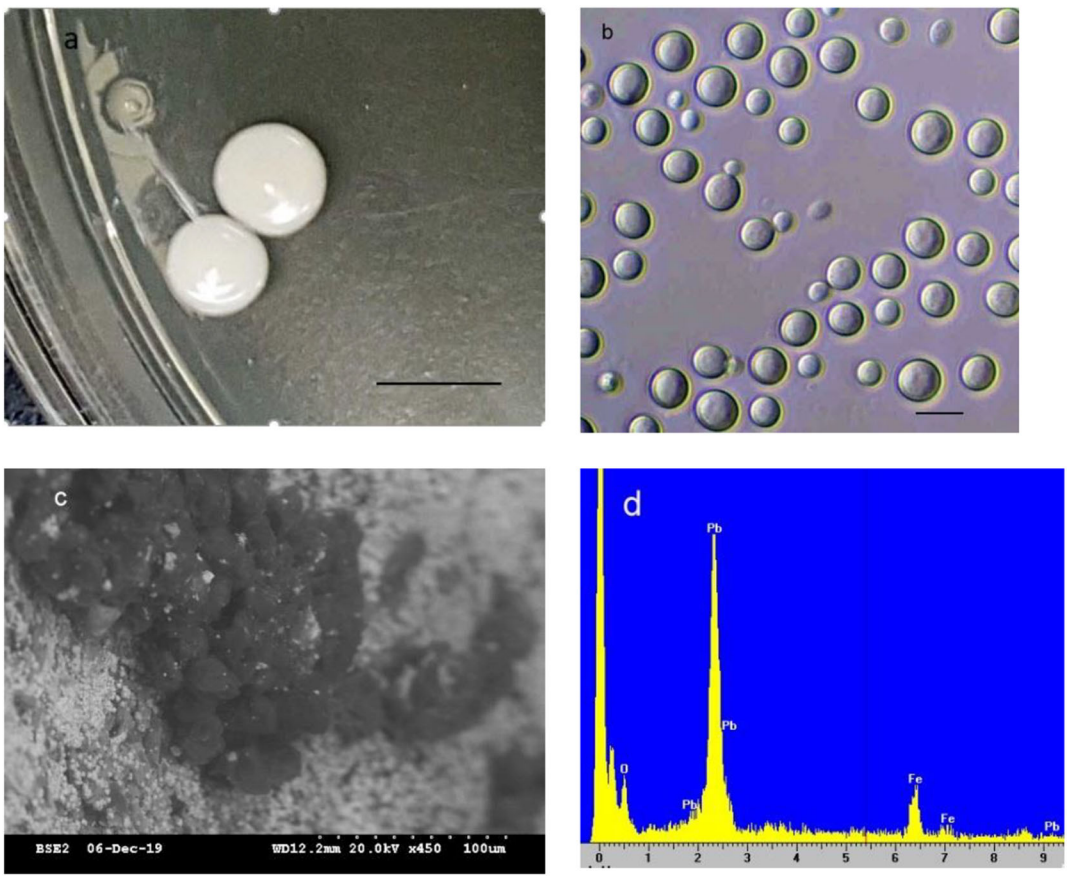

Figure 3. 3a Wallachia Colonies, bar = 3mm: 3b yeast cells, bar = 10μm: 3c cork surface showing possible yeast cells: 3d X ray profile of cork surface indicating presence of lead and iron.

Culture plating of samples from bottle W2 failed to produce growth on any media. Metagenomic profiling of DNA extracted from bottle W2 indicated the presence of some of the same fungi and bacteria as identified in bottle W1. The predominant fungi and bacteria were those typically associated with beer – *Brettanomyces, Debaryomyces* yeast and lactobacilli and pediococci. The absence of typical marine bacteria such as *Pseudomonas* and *Vibrio* species suggests that although there was dilution by sea water, ingress of microroganisms was limited, possibly due to filtration through the cork which remained secure. Moreover, eight of the 12 species of bacteria identified in beer from W2 were also found in beer from W1 suggesting that the sample can be regarded as representative.

Beer from bottle W3 produced colonies on agar after five days of incubation. Two yeast species were cultured and identified

Table 2. Proportion of species identified from Wallachia W1. Values indicate species with >1% proportion. Additional bacterial identifications at <1% proportion listed in S2. sp – single unidentified species of genus, spp – multiple unidentified species of genus.

Fungal species	%	Characteristics
Aspergillus sp.	35.88	Common airborne mould
Brettanomyces/Dekkera bruxellensis	18.76	Known brewing yeast
Debaryomyces hansenii	19.44	Known fermentation yeast
Metschnikowia pulcherrima	14.66	Fruit and flower yeast
Stereum sanguinolentum	11.15	Conifer wood saprophyte
Bacterial Species	**%**	Characteristics
Acetobacterium tundrae	1.10	Beer spoilage bacterium
Acidaminococcus spp.	1.62	Gram negative bacterium
Actinobaculum urinale	2.27	Gram negative anaerobic bacterium
Bacillus licheniformis	1.45	Plant and soil bacterium
Bacteroides sp.	3.96	Opportunistic pathogen
Finegoldia magna	7.59	Commensal skin bacterium
Fusobacterium sp.	2.47	Possible pathogenic bacterium
Kocuria rosea	1.26	Possible urinary tract pathogen
Lactobacillus spp.	1.39	Common beer spoilage bacterium
Lactococcus lactis	1.56	LAB common in dairy products
Mogibacterium pumilum	2.34	Possible oral cavity bacterium
Paucilactobacillus vaccinostercus	32.01	LAB associated with fermented foods
Pediococcus damnosus	10.75	Common beer spoilage bacterium
Pediococcus inopinatus	11.08	Common beer spoilage bacterium
Shigella sonnei	2.46	Enteric pathogen
Staphylococcus epidermidis	3.26	Commensal skin bacterium
Stenotrophomonas maltophilia	1.96	Soil bacterium
Varibaculum cambriense	1.18	Possible pathogenic bacterium

Table 3. Proportion of species identified from Wallachia W2. Values indicate species with >1% proportion. Additional fungal and bacterial identifications at <1% proportion listed in S2. sp – single unidentified species of genus, spp – multiple unidentified species of genus.

Fungal species	%	Characteristics
Brettanomyces/Dekkera bruxellensis	78.67	Known brewing yeast
Debaryomyces coudertii	1.93	Avian associated yeast
Debaryomyces hansenii	19.39	Known fermentation yeast
Bacterial species		
Aminomonas spp.	2.01	Amine digesting anaerobe
Chroococcidiopsis sp.	5.28	Cyanobacteria
Cyanothece spp.	1.65	Marine photosynthetic bacterium
Desulfobacter psychrotolerans	1.55	Psychrotolerant sulphate reducer
Fusobacterium sp.	9.21	Gram negative bacilli
Fusobacterium spp.	2.88	Gram negative bacilli
Kocuria rhizophila	0.22	Soil actinomycete
Parabacteroides distasonis	1.65	Common intestinal bacterium
Paucilactobacillus vaccinostercus	17.56	LAB associated with fermented foods
Pediococcus damnosus	54.74	Common beer spoilage bacterium

by ITS sequencing as *Brettanomyces/Dekkera bruxellensis* and *Debaryomyces hansenii* with a 99% similarity to the *Debaryomyces hansenii* isolated from W1. In addition, a further 46 bacterial species were identified by metagenomic profiling including *Lactobacillus plantarum (*reclassified as *Lactiplantibacillus plantarum)* and *Pediococcus damnosus*. Full details of the fungi and bacteria species with proportion of reads <1% is reported in supplementary information Table S2.

Kings Ale 1902

Chemical analysis of beer characteristics of alcohol by volume, specific gravity, colour, pH and bitterness are reported in Table 5 for the two King's Ale samples. Sediment from the bottles indicated the presence of yeast cells (Figure 4a). Colonies from bottle K2 grew on the aerobic lysine and WLN agar and were subcultured for purification and further investigation. Microscopy indicated

Table 4. Proportion of species identified from Wallachia W3. Values indicate species with >1% proportion. Additional fungal and bacterial identifications at <1% proportion listed in S2. sp – single unidentified species of genus, spp – multiple unidentified species of genus.

Fungal species	%	Characteristics
Acremonium charticola	19.41	Food fungus
Brettanomyces/Dekkera bruxellensis	27.27	Known brewing yeast
Debaryomyces hansenii	6.23	Known fermentation yeast
Hyphodontia sambuci	2.98	Timber parasite fungus
Ogataea candida boidinii	2.92	Environmental and food yeast
Phialotubus microsporus.	8.01	Environmental fungus
Saccharomyces byanus	20.18	Known wine yeast
Saccharomyces cerevisiae	2.73	Known brewing yeast
Trechispora sp.	7.57	Bark associated mould
Bacterial species		
Acetobacterium tundrae	12.53	Psychrophilic bacterium
Aminomonas spp.	4.10	Amine digesting anaerobe
Bacillus amyloliquefaciens	1.00	Soil bacterium
Bacillus subtilis	3.32	Common soil bacterium
Corynebacterium kroppenstedtii.	5.57	Lipophilic *Corynebacterium*
Desulfovibrio sp.	8.69	Sulphate reducing bacterium
Gamella sp.	3.05	Pressure resistant bacterium
Gluconacetobacter liquefaciens	2.10	Beer spoilage bacterium
Halomonas xinjiangensis	8.40	Salt resistant bacterium
Leuconostoc citreum	8.18	Food fermenting LAB
Marinilabilia sp.	6.75	Marine lipolytic bacterium
Oscoillospora spp.	1.02	Clostridial bacterium
Secundilactobacillus malefermentans	30.99	Beer spoilage LAB
Stenotrophomonas maltophilia	1.45	Environmental bacterium
Xanthomonas sp.	1.11	Plant pathogen

Table 5. Chemical analysis of beer from King's Ale bottles.

Sample	ABV %	Original gravity	Present gravity	pH	Acidity % (v/v) as lactic acid	Colour EBC	Bitterness EBU
KA1	10.42	1.120	1.040	4.04	0.567	215.5	54.7
KA2	11.36	1.130	1.043	4.02	0.576	217.0	54.7

that the cells were yeast (Figure 4b). DNA extraction and ITS sequencing confirmed these to be *Saccharomyces cerevisiae* with 99.0% identity across 720 nt aligned in BLASTn. Metagenomic profiling of DNA from the sediment of the bottles indicated a range of microorganisms as detailed in Table 6 and including *Brettanomyces* and *Debaryomyces* species.

Discussion

The primary question addressed by this study was whether analysis of intact, historic beer samples can provide details of beer components and their microbiology?

Chemical analysis of Wallachia and King's ale beers provided relevant information of beer character showing that these two beers were close to style expectations – a stout and a barley wine respectively. Both were high in alcohol, 7.5% for the Wallachia beer and 10-11% for the King's ale. Analysis of nine UK stouts reported to the Institute of Brewing in 1908 noted an average ABV of 6.56% ± 0.73% indicating the Wallachia beer to be close to contemporary brands *(20)*. Final gravities were high for the King's Ale beers at 1.040 reflecting the strong initial worts (original gravity 1.125) and suggesting limited digestion of dextrins during fementation and maturation and thus a strong mouthfeel. Final gravities of the Wallachia beers (1.007) were more in line with expectations of standard fermentations. Colour was high but bitterness low for the Wallachia stout as would be typical of the style at the time. In contrast colour was low but bitterness high for the King's ale. The King's ale is reported to have been brewed as a keeping beer and would have relied on alcohol and hops as preservatives *(30)*. Further analysis may provide information on the character of the hop components of the beers and other flavour compounds.

The microbiology of these beers is particularly interesting as the presence of a wide range of fungi and bacteria were suggested by metagenomic profiling. The identification of *Brettanomyces/Dekkera bruxellensis*, and *Saccharomyces* species as components of the fungal microbiota in the Wallachia beer and in the King's ale samples agrees with contemporary understanding of these species in Victorian stock ale *(16)*. The limited presence and variability of *Saccharomyces* species in the Wallachia beer and King's Ale samples is interesting. Analysis of ancient DNA does, inevitably,

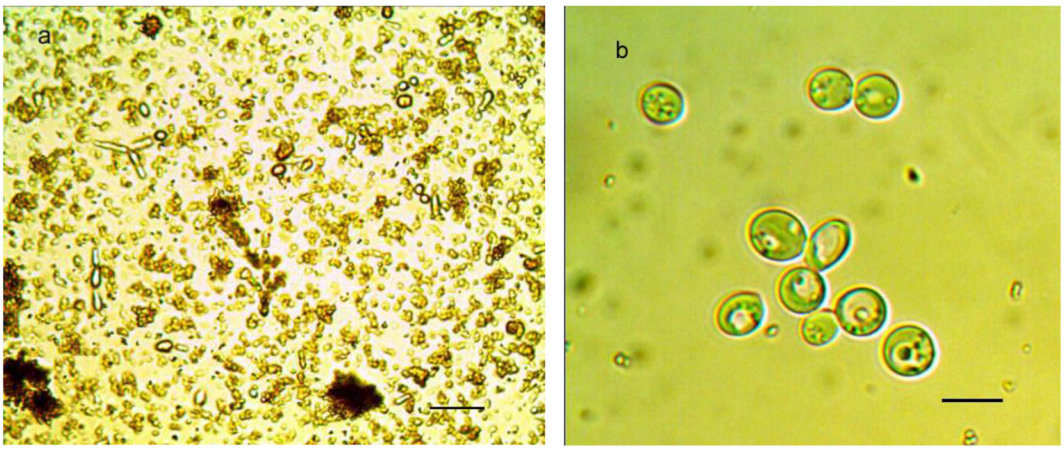

Figure 4. King's Ale isolates. 4a Bottle sediment, bar = 40 μm: 4b Cells of *Saccharomyces cerevisiae*, bar = 10 μm:

Table 6. Proportion of species identified from Kings Ale bottles 1 and 2. sp – single unidentified species of genus, spp – multiple unidentified species of genus.

Fungal species			
King's Ale 1	**%**	**King's Ale 2**	**%**
Brettanomyces/Dekkera bruxellensis	0.046	*Aspergillus* sp.	59.079
Debaryomyces cordertii	0.004	*Aspergillus versicolor*	6.268
Debaryomyces hansenii	0.004	*Candida* sp.	0.1495
Talaromyces rotundus	99.947	*Debaryomyces hansenii*	9.483
		Dioscorea alata	0.153
		Malassezia restricta	0.370
		Penicillium sp.	0.019
		Sacchromyces boulardii	0.142
		Saccharomyces cerevisiae	0.265
		Saccharomyces pastorianus	0.632
		Talaromyces rotundus	24.440
Bacterial species			
King's Ale 1	**%**	**King's Ale 2**	**%**
Bacillus subtilis	1.753	*Anabaena* spp.	0.020
Bradyrhizobium lupini	0.625	*Azospirillum* spp.	2.802
Bradyrhizobium sp	5.147	*Bacillus subtilis*	0.118
Kocuria rhizophila sp.	0.003	*Corynebacterium tuberculostearicum*	0.017
Staphylococcus aureus	0.100	*Cyanothece* spp.	0.017
Staphylococcus epidermidis	0.227	*Halospirulina* sp.	44.976
Staphylococcus equorum	88.032	*Hyphomonas* spp.	0.768
Staphylococcus haemolyticus	0.148	*Staphylococcus aureus*	0.037
Staphylococcus pettenkoferi	0.113	*Staphylococcus equorum*	1.557
Staphylococcus sp.	1.828	*Staphylococcus haemolyticus*	0.014
Staphylococcus spp.	0.182	*Staphylococcus* sp.	0.013
Staphylococcus xylosus	0.131	*Stenotrophomonas maltophilia*	2.487
Streptococcus salivarius	1.711	*Stenotrophomonas* sp.	0.464
		Stenotrophomonas spp.	9.828
		Turicella sp.	1.855

carry limitations and variabilities due to the degradation of the DNA and the shortness of molecules produced affecting PCR productivity *(34)*. However, it is likely that the cold and dark conditions in shipwreck samples will be more suitable for preservation than storage at higher temperatures. These conditions are also conducive for survival of vegetative cells and spores so allowing recovery of viable cultures. Analysis of bacteria from historic dental calculus samples has noted the possibility of selective identification of some species due to shorter 16S rRNA amplicons being disproportionately amplified *(35)*. There is no comparable study to suggest that ribosomal DNA fragments from *Saccharomyces* species are at similar risk, but further analysis would be relevant *(35)*. The

detection of *Saccharomyces* species in W3 and KA2 suggests that this is an unlikely explanation. Future analysis of the extracted DNA, however, would be useful to determine signatures of degradation expected of ancient DNA and other markers of *Saccharomyces*.

The extensive presence of *Debaryomyces* in all the beer samples is interesting as this genus has not been noted as a feature of historic brewing but has been identified in spontaneous fermentations, for example in Belgian lambic beers *(36,37)*. It is also, well recognised in various food fermentations *(38)* with strong osmophilic properties and can have the capacity to ferment wort sugars *(39)*. Although the genus was reported to the brewing industry in 1909 *(40)* it has not featured as a major contributor to beer fermentations but was recently included in a screening of non-*Saccharomyces cerevisiae* strains for potential brewing use *(41)*. It is interesting that recent research by De Roos et al *(42)* has identified *Debaryomyces* as a component of the microbiota of cask surfaces used in lambic brewing.

A wide range of bacteria were identified in the Wallachia beer samples although the greatest proportion were lactic acid bacteria, specifically lactobacilli and pediococci. It is relevant that studies on bottled stout in 1908 indicated the regular presence of bacteria purported to be 'of a lactic type' and given the name *Saccharobacillus pastorianus (20)*. The identification of staphylococci species in the Wallachia samples and more extensively in both King's Ale beers suggests environmental contamination, possibly from brewery workers but also, potentially in the case of *Staphylococcus equorum,* from horses *(43)*. Horses would have been active in brewery operations, but the species is also associated with fermented foods *(44)*. Other environmental contaminants in these beers included *Aspergillus* and *Penicillium* mould species which are common air contaminants, and which were recognised by 1889 as readily growing in brewery environments *(17, 19)*.

The successful cultivation of yeast from historic bottled beer is encouraging and suggests a valuable resource exists for today's brewing. At the moment it is impossible to say whether vegetative cells or spores were the surviving structure but the conditions in shipwreck samples would limit stress. The potential survival of spores of non-*Saccharomyces* yeast in other samples of historic beers has been suggested *(4)*. Yeast species do have stress resistance mechanisms which could account for survival in low nutrient conditions *(45)* and it will be interesting to test these with the extracted samples.

With the isolations made from these samples and the metagenomic profiles obtained, is it possible to provide information to direct authentic recreations of past beers? The chemical information suggests directions to recipe formulation albeit with some limitations in that alcohol may be metabolised and bitterness decline. Acidity and pH measurements suggest that there may have been acidification in the Wallachia beer but less so in the King's ale. Colour may also fade although entrapment in shipwreck conditions will limit photolysis. The metagenomic profiles do suggest that these beers had a range of microorganisms in addition to their brewing yeast, particularly lactic acid bacteria in the Wallachia stout. This would be expected in such brewing conditions and suggests that brewing microbiology may have been more varied than previously considered. The more limited yeast hygiene practiced at the time would also have led to a greater fungal and bacteria microbiota persisting in the beers.

One particularly interesting question is why *Saccharomyces* species were only present as a small proportion in some samples of the beers. *Saccharomyces* are major colonisers of natural fermentations *(37)* and would be expected to be identified in the samples. The predominance of *Brettanomyces* species in the Wallachia beer agrees with the identification of these yeasts as a critical component of British Stock ale *(15, 46)*. A similar predominance of *Debaryomyces* in the Wallachia beers is particularly interesting and its presence in a King's ale bottle suggests that it may be more of a common feature than previously recognised. In this context, it is relevant that in other studies, a yeast isolated from Iron age Philistine jugs commonly associated with beer was identified as a *Debaryomycete* strain *(8)*. Similarly, a sample of 1825 Porter beer isolated from a shipwreck bottle in 1987 was also a *Debaryomyces* species (https://www.brewlab.co.uk/the-original-flag-porter-story/). It is possible then that the presence of *Debaryomyces* species in both the Wallachia and King's ale samples suggests it was a more prevalent species in historic brewing and worthy of consideration for future brewing studies.

Further analysis of the characteristics of the yeasts isolated here is ongoing to determine their suitability for fermentation and potential beverage production as conducted with other ancient yeasts *(8)* and bacteria *(29)*. The potential of historic beers directly influencing future brewing is increasingly possible.

Author contributions

Keith Thomas: Conceptualisation, methodology, formal analysis, investigation, writing – original draft and review.
Kayleigh Ironside: Methodology, investigation, writing – review and editing.
Lisa Clark: Investigation.
Lewis Bingle: Methodology, formal analysis, writing – review and editing.

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Conflict of interest

Keith Thomas is a Director of Brewlab Ltd. The other authors declare there are no conflicts of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.