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A special drop: Characterising yeast isolates associated with fermented beverages produced by Australia's Indigenous peoples

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ABSTRACT

Way-a-linah, an alcoholic beverage produced from the fermented sap of *Eucalyptus gunnii*, and *tuba*, a fermented drink made from the syrup of *Cocos nucifera* fructifying bud, are two of several fermented beverages produced by Australian Aboriginal and Torres Strait people. Here we describe the characterisation of yeast isolates from samples associated with the fermentation of *way-a-linah* and *tuba*. Microbial isolates were obtained from two different geographical locations in Australia - the Central Plateau in Tasmania, and Erub Island in the Torres Strait. While *Hanseniaspora* species and *Lachancea cidri* were the most abundant species in Tasmania, *Candida* species were the most abundant in Erub Island. Isolates were screened for tolerance to stress conditions found during the production of fermented beverages and for enzyme activities relevant to the appearance, aroma and flavour of these beverages. Based on screening results, eight isolates were evaluated for their volatile profile during the fermentation of wort, apple juice and grape juice. Diverse volatile profiles were observed for beers, ciders and wines fermented with different isolates. These findings reveal the potential of these isolates to produce fermented beverages with unique aroma and flavour profiles and highlight the vast microbial diversity associated with fermented beverages produced by Australia's Indigenous peoples.

INTRODUCTION

Australia is proudly home to one of the oldest continuous cultures on the planet. As such, it is not surprising that grinding-stones and starch residues have been found in Cuddie Springs in north-west New South Wales and in Arnhem Land in the Northern Territory, dated to be at least 30,000 (Fullagar and Field, 1997) and 65,000 years old (Clarkson et al., 2017), respectively. These stones were used to grind wild seeds into flour, which in turn was baked as bread, placing Indigenous Australians as the world's first bakers. The same may be true for fermented beverages. Before the arrival of the first Europeans, Aboriginal people produced several fermented drinks. In Western Australia, sugar-rich bottlebrush and cones of *Banksia* flowers were used to make *gep* or *mangaitch*; in Tasmania, *Eucalyptus* tree sap was used to

make *way-a-linah*; while in the Northern Territory, *kambuda* was made from crushed nuts of the palm-like Pandanus tree (Brady, 2008; Joondalup, 2010). In the Torres Strait, islanders produced *tuba* from the fructifying buds of coconut palms and distilled this beverage into a strong spirit, a knowledge gained from Southeast Asian traders (Brady and McGrath, 2010).

Eucalyptus gunnii, commonly known as cider gum, is a tree species endemic to Tasmania, which is mainly found in cold, waterlogged habitats, such as lake edges, or poorly drained valley flats (Williams and Potts, 1996). This tree produces a sweet sap that often flows from naturally occurring holes in the trunk and accumulates at the base of the tree and/or on the soil, where it spontaneously ferments (Varela et al., 2020). Tasmanian *Palawa* people call this drink *way-a-linah* and used stone tools to bore a hole in the trunk of the tree and to make a larger depression at the base to collect the sap flow, which was then left to ferment after covering the collecting hole with a flat stone (Brady, 2008).

Tuba was made from the syrup that seeps from a cut made in the unopened fructifying bud of *Cocos nucifera*, commonly known as coconut tree (Brady and McGrath, 2010). Over several days the unopened bud, usually pointing upwards, would be bound with a string and gradually forced to point down using ropes. The fructifying bud would then be cut and a receptacle placed underneath to collect the syrup. People would climb the tree every day to collect the sap, prune the flowers and place another container. The collected sap was left to ferment for several days. Neither *way-a-linah* or *tuba* are regularly produced today by Australia's Indigenous peoples and first-hand knowledge of traditional indigenous Australian fermentation practices has been lost.

Numerous microorganisms, including fungi, yeast and bacteria, which are present on the surface of fruits and grains, inside fermentation pots or inadvertently introduced by human action, are responsible for the conversion of sugar into alcohol and for the many secondary metabolites that shape the aroma and flavour of fermented beverages (Jolly et al., 2014;

Varela, 2016). Some yeasts are particularly suited to the process of alcoholic fermentation and rapidly become the dominant group of microorganisms as fermentation progresses. *S. cerevisiae*, also known as baker's yeast, is the main species associated with brewing and winemaking, however numerous non-*Saccharomyces* yeast species also play key roles during the production of many fermented beverages (Varela, 2016).

These non-*Saccharomyces* species are being increasingly studied as a potential source of diverse, novel enzymes that can improve or provide novel characteristics to fermented beverages (Belda, Ruiz, et al., 2016). These traits include physical appearance, for instance turbidity and foam production, colour, aroma, and flavour (Belda, Conchillo, et al., 2016; Canonico et al., 2016; Daenen et al., 2008; Hu et al., 2016; Ribeiro et al., 2015; Segura-Garcia et al., 2015). In addition, non-*Saccharomyces* yeasts can potentially enhance the regional identity of fermented beverages. Although this has only been explored for winemaking (Garofalo et al., 2016; Lopandic et al., 2007; Parapouli et al., 2010), the use of regional yeast isolates could help differentiate the final product and offer a good alternative for consumers conscious about local production and identity (Varela, 2016).

In this work, we describe the characterisation of yeast isolates from samples associated with the fermentation of *way-a-linah* in the Central Plateau in Tasmania and of *tuba* in Erub Island in the Torres Strait. Microbial isolates showing tolerance to common stress conditions found during the production of fermented beverages, as well as enzyme activities relevant to the appearance, aroma and flavour of these beverages, were evaluated for their ability to conduct fermentations of substrates for beer, cider and wine and the resulting volatile profiles examined.

MATERIALS AND METHODS

Sampling

Samples were obtained from two different geographical locations in Australia, the Central Plateau in Tasmania, and Erub Island in the Torres Strait. In Tasmania, bark, sap and soil samples were collected from the cider gum *E. gunnii* as described previously (Varela et al., 2020), while in Erub Island samples were collected from flowers, fruits and plants using sterile, cotton-tipped swabs moistened with sterile saline solution (NaCl 0.9 %). Details for samples collected in Tasmania and Erub Island are listed in Tables S1 and S2, respectively. Samples were plated onto YPD agar (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L, agar 15 g/L) and Wallerstein Laboratory nutrient agar (Amyl Media Pty Ltd, Dandenong, Australia) and incubated at 28 °C for four days. Individual colonies were picked manually, arrayed in a 96-well format, which included a *S. cerevisiae* control strain (EC1118) in each plate, and kept as glycerol frozen stocks at – 80 °C.

Colony identification

Yeast colonies were identified using amplicon sequencing. Arrayed colonies were resuspended in sterile water and boiled for 10 min to release DNA. This DNA suspension was then subjected to a two-step PCR process that amplifies the ITS2 locus from the fungal ribosomal internal transcribed spacer (ITS) region while adding both custom in-line barcodes and sequences necessary for Illumina sequencing (including compatible Illumina dual-indexes) using the BITS/B58S3 primer set (Bokulich and Mills, 2013). Sequencing was performed using 2 × 300 bp chemistry (Ramaciotti Centre for Functional Genomics, Australia). Following sequencing, paired-end reads were quality trimmed [Trimmomatic v0.38, (Bolger et al., 2014)], adaptor trimmed [Cutadapt v1.16, (Martin, 2011)] and merged into single synthetic reads [FLASH2 v2.2.00, (Magoc and Salzberg, 2011)]. Merged reads were de-replicated [USEARCH v10.0.240, (Edgar, 2010)] and clustered [Swarm v2.2.2, (Mahe et al., 2014)] into operational taxonomic units (OTUs) as presented previously (Sternes et al., 2017). Taxonomic annotation was performed against the UNITE database (qiime_ver8_dynamic_02.02.2019)

using a 98% similarity cut off, assign_taxonomy.py module of QIIME v1.9.1 (Caporaso et al., 2010). Sequences for all fungal OTUs identified in this study are listed in Table S3.

Colony phenotyping

Frozen stocks with arrayed colonies were pinned in duplicate on YPD agar plates using a Tecan Freedom EVO 150 robotic platform with a 96-pin stainless steel pin tool (Tecan Group Ltd, Männedorf, Switzerland) and incubated at 28 °C for 3 days. YPD colonies were then pinned onto synthetic complete (SC) agar medium (20 g/L of glucose, 1.7 g/L yeast nitrogen base without ammonium sulfate or amino acids, 5 g/L ammonium sulfate and 20 g/L agar) and grown at 28 °C for 5 days. These plates were used as source plates for screening colonies under different environmental conditions and to assess key enzyme activities. For ethanol tolerance, SC source plates colonies were pinned onto SC agar media containing 3%, 6% or 12% (v/v) ethanol. For osmotic stress, source plates colonies were pinned onto SC agar media containing either 20% or 40% (w/v) glucose. For copper tolerance, source plates colonies were pinned onto SC agar media containing 0.2 mM copper sulfate. Growth under anaerobic conditions was screened by pinning source plate colonies onto SC agar media followed by incubation in a Type B Vinyl chamber anaerobic hood (Coy Labs, Michigan, USA). Growth at low temperature was screened by incubating pinned SC agar plates at 12 °C. Sulfite tolerance was assessed by pinning onto SC agar plates containing 4.5 g/L tartaric acid and 1 mM sodium sulfite (Naumov et al., 2013), which resulted in 13 mg/L free SO₂ or 0.4 mg/L molecular SO₂. Hydrogen sulfide (H₂S) production was assessed in SC agar media containing copper, since H₂S reacts with copper generating CuS, which turns colonies brown. Agar plates were incubated at 28 °C for 5 days and then photographed using the PhenoBooth (Singer Instruments, Somerset, UK). Photos were used to quantify colony sizes and measure colony colour with the software PhenoSuite v2.20.504.1 (Singer Instruments, Somerset, UK).

The sensitivity of isolates to environmental conditions was quantified by expressing the log₂ ratio of the colony size for each test condition over the colony size on SC agar medium. For

sulfite tolerance, the log₂ ratio was obtained from the colony size in media with sodium sulfite over the colony size on SC media containing tartaric acid. H₂S production was expressed as the log₂ ratio of the colony brightness in SC media over the colony brightness on media containing copper.

Five different enzyme activities were assessed including cellulase, pectinase, protease, β -lyase and β -glucosidase. Colonies from SC source plates were inoculated into 96-well plates containing 200 μ L of SC liquid media in each well. Liquid source plates were incubated at 28 °C for 2 days and then used to inoculate 96-well plates containing modified SC medium, incubated at 28 °C for 3 days and assessed as follows. For cellulase activity, 96-well plates contained SC medium plus CMC (1 g/L carboxymethyl cellulose) with cellulase activity measured spectrophotometrically with Congo Red at 530 nm (Haft et al., 2012). SC medium plus PGA (1.5 g/L polygalacturonic acid) was used for pectinase activity, which was determined spectrophotometrically (535 nm) with ruthenium red (Ortiz et al., 2014). Protease activity was determined spectrophotometrically (430 nm) with trichloroacetic acid in cultures grown in SC medium with 2.5 g/L azocasein (Lario et al., 2015). For β -glucosidase activity, source cultures were grown in SC medium with cellobiose, and β -glucosidase activity determined spectrophotometrically (400 nm) with p-nitrophenyl- β -D-glucopyranoside (PNPG) (Schmidt et al., 2011). β -Lyase activity was measured spectrophotometrically (310 nm) from the release of 2-mercaptothiazole by cultures grown in SC medium with TAC (1 g/L S-(2-thiazolyl)-L-cysteine) and 12 g/L yeast carbon base (Cordente et al., 2022). All enzyme activities were expressed as the log₂ ratio of the absorbance value for each activity over the absorbance value for uninoculated media.

In both the case of growth assays and enzyme assays, the differences between strains were more easily visualised by converting log₂ ratios to discrete values (scores from 1 to 4) based on the log₂ ratios of a reference. For agar plates, the *S. cerevisiae* control strain EC1118 a

widely used wine strain, was employed as the reference, while for enzyme assays uninoculated media was used as the reference. Positive attributes scored higher, that is, colonies with log₂ ratios between 75-100% of the log₂ ratio of the *S. cerevisiae* control were given a score of 4, colonies with ratios between 50-75% were given a score of 3, colonies with ratios between 25-50% were given a score of 2, whereas colonies with ratios lower than 25% were given a score of 1. For H₂S production, colonies with log₂ ratios between 100-120% of the log₂ ratio of the *S. cerevisiae* control were given a score of 4, colonies with ratios between 120-130% were given a score of 3, colonies with ratios between 130-150% were given a score of 2, whereas colonies with ratios higher than 150% were given a score of 1. For cellulose, pectinase and protease activities, cultures with log₂ ratios higher than 150% of the log₂ ratio for the uninoculated media were given a score of 4, cultures with ratios between 125-150% were given a score of 3, cultures with ratios between 110-125% were given a score of 2, whereas cultures with ratios lower than 110% were given a score of 1. For β -glucosidase and β -lyase activities, cultures with log₂ ratios four-times higher than the log₂ ratio for the control were given a score of 4, cultures with ratios between three- and four-times higher were given a score of 3, cultures with ratios between two- and three-times higher were given a score of 2, whereas cultures with ratios lower than twice the log₂ ratio for the control were given a score of 1.

Fermentation screening

Selected strains were evaluated for fermentation potential in defined GFM media containing 40 g/L glucose, 40 g/L fructose, 40 g/L maltose, 1.7 g/L YNB without ammonium sulfate or amino acids and 5 g/L ammonium sulfate. Starter cultures were grown overnight in YPD medium under aerobic conditions at 28 °C (200 rpm) and then inoculated to OD₆₀₀ 0.1 (approximately 1 x 10⁶ cells/mL) in GFM media diluted 1:1 with sterile water. Starter cultures were incubated (28 °C, 200 rpm) for two days and then used to inoculate ferments to OD₆₀₀ 0.1, which were magnetically stirred (120 rpm) and incubated at 28 °C for 14 days after which

fermentation was stopped. Fermentations were performed in duplicate using a high throughput robotic fermentation system (van Holst Pellekaan et al., 2021) in flasks containing 90 mL of media. Post fermentation, flasks were kept at 4 °C for five days and then sampled for metabolite analysis.

Similarly to the colony phenotyping data, metabolite concentrations were used to estimate log₂ ratios, which were converted to discrete scores based on the log₂ ratios of the *S. cerevisiae* reference. Positive attributes received higher scores, thus for sugar, higher scores indicate high consumption, for citric, succinic, and lactic acids, glycerol and ethanol, higher scores indicate higher production, whereas for acetic acid higher scores indicate low production.

Beer, cider and wine fermentations

Selected colonies were evaluated for their ability to ferment wort, apple juice and grape juice and produce beer, cider and wine, respectively, in sequential fermentation with *S. cerevisiae*. Fermentations were performed in triplicate using a high throughput robotic fermentation system (van Holst Pellekaan et al., 2021) in flasks containing 90 mL of medium and a 2-step starter culture preparation. Starter cultures were first grown overnight in YPD medium under aerobic conditions (28 °C, 200 rpm), and then inoculated to 5 x 10⁶ cells/mL into wort, apple juice or grape juice, diluted 1:1 with sterile water. Following incubation at 22 °C (200 rpm) for two days, these starter cultures were then used to inoculate ferments to 5 x 10⁶ cells/mL. Ferments were magnetically stirred (120 rpm) at 22°C for 14 days. After 50% of sugar was consumed (estimated by weight loss in a preliminary experiment), ferments were sequentially inoculated with a *S. cerevisiae* control strain (5 x 10⁶ cells/mL). A control fermentation inoculated solely with a *S. cerevisiae* control strain was also performed. Control strains included: Safale US-05 (Fermentis, Marcq-en-Baroeul, France) for beer, Safcider AB-1 (Fermentis, Marcq-en-Baroeul, France) for cider and Lalvin EC1118 (Lallemand, Montreal, Canada) for wine. Red ale malt extract (62 g/L sugar, Coopers Brewery, Adelaide, Australia)

and apple juice (97 g/L sugar, Berri Juice, Melbourne, Australia) were purchased from Woolworths (Adelaide, Australia), whereas Muscat Gordo Blanco juice (233 g/L sugar) was obtained from the Yalumba winery (Angaston, Australia). initial concentration of carbohydrates or Apple juice and grape juice were adjusted to 200 mg/L of yeast assimilable nitrogen with the addition of diammonium phosphate. After fermentations were stopped, flasks were kept at 4°C for five days and then sampled for analysis.

Chemical analysis

Quantification of sugars (glucose, fructose, maltose, maltotriose), organic acids (citric, succinic, lactic, acetic acids), glycerol and ethanol was performed by HPLC using a BioRad HPX87H column at 65°C with H₂SO₄ 5 mM at 0.5 mL/min (Varela et al., 2004). Analysis of higher alcohols, and acetate- and ethyl- esters was performed using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME/GCMS), with polydeuterated internal standards for stable isotope dilution analysis (Varela et al., 2017). Non-chromatographic volatile fingerprinting was performed by Metabolomics Australia (Adelaide) using GCMS as previously described (Bizaj et al., 2012).

Data and statistical analyses

Assessment of chemical composition was undertaken using ANOVA and Tukey's multiple comparisons test (alpha = 0.05) using the functions aov and HSD.test from the R packages stats (Team, 2017) and agricolae (de Mendiburu and Yaseen, 2020), respectively, in R version 3.6.2 (Team, 2017). Principal component analysis (PCA) was performed to reduce the dimensionality of data and to visualise differences between samples. Data was normalised to unit variance using the PCA function from the R package FactoMineR (Lê et al., 2008). Hierarchical clustering was performed and visualised using the R package heatmap3 (Zhao et al., 2021).

RESULTS

Microbial isolates were obtained from two different geographical locations in Australia - the Central Plateau in Tasmania, and Erub Island in the Torres Strait, approximately 3,600 km apart (Figure 1). While Tasmania is where *way-a-linah* was originally made, to our knowledge Erub Island is one of the last reported places to produce *tuba* in Australia. In Tasmania, bark, sap and soil samples were collected from the cider gum *E. gunnii*, while in Erub Island, samples were collected not only from palm tree inflorescences and coconuts, but also from other flowers, fruits and plants, including hibiscus and frangipani flowers, bananas, paw paws and other native fruits.

Microbial diversity of isolates and phenotyping

A total of 1029 isolates from Tasmania and 71 isolates from Erub Island were identified by ITS phylotyping (Table 1). The higher number of isolates in Tasmania is due to a larger number of samples (84 samples in Tasmania vs 43 samples in Erub Island), an increased number of colonies obtained from each sample (12 colonies per sample in average in Tasmania vs 4.3 colonies per sample in Erub Island), and greater number of identified fungal OTUs (63% of all isolates in Tasmania vs 36% in Erub Island). The most abundant species isolated from Tasmania were *Hanseniaspora valbyensis*, *Lachancea cidri* and *Hanseniaspora osmophila*, whereas in Erub Island *Candida* species, including *C. stellimalicola*, *C. intermedia* and *C. tallmaniae*, were the most abundant (Figure 1). Given the large number of strains found for some species, a preselection of isolates was made to maximise microbial diversity, while reducing the overall number of isolates to be studied (Table 1). Thus, for species with less than 30 individual isolates, all colonies were selected. Whereas for species with high number of isolates, these were randomly selected from the different OTUs identified within each species. A total of 194 isolates (144 from Tasmania and 50 from Erub Island), were screened for their tolerance to common stress conditions found during the production of fermented beverages and for enzyme activities relevant to the appearance, aroma and flavour of these beverages. Colony sizes and enzyme activities were converted to discrete scores to clearly

visualise differences between isolates. The ability to grow under different stress conditions, the presence of enzyme activities and a low H₂S production were considered positive attributes and had high scores.

Scores for the screened isolates are shown in Table S4, whereas a heatmap with hierarchical clustering results is illustrated in Figure S1. Six clusters were identified, with isolates from Tasmania and Erub Island including *L. cidri* and *Candida* species in the first cluster. These isolates showed tolerance to ethanol at 3% and 6%, most were tolerant to osmotic and copper stress and produced low H₂S. All Tasmanian isolates in this cluster grew in the absence of oxygen but were sensitive to SO₂. On the contrary most isolates from Erub Island in this cluster were unable to grow anaerobically but were tolerant to SO₂. Only a few isolates in this cluster showed some enzyme activities, particularly protease and β -lyase activities. The second cluster included *H. osmophila*, *Candida*, *Rhodotorula* and *Debaryomyces* species. Many of these isolates were tolerant to ethanol 3%, but most did not grow at 6% ethanol. Most isolates in this cluster showed some protease and β -lyase activities. The third cluster comprised mainly Tasmanian isolates, including *Hanseniaspora* species, *L. cidri* and other minor species. Most of these isolates were sensitive to osmotic stress, copper and SO₂, showed high H₂S production and did not have any enzyme activities. The fourth cluster included mainly *H. valbyensis* isolates, some *H. osmophila* and *L. cidri* strains and other minor species, all from Tasmania. Most of these isolates were sensitive to SO₂, showed high H₂S production and had protease activity. The fifth cluster included mainly isolates from Erub Island, with most being sensitive to copper, anaerobic conditions, and low temperature, but having β -lyase and β -glucosidase activities. The last cluster comprised *A. pullulans* isolates from Erub Island, which were sensitive to ethanol, anaerobic conditions and SO₂, and had cellulase, pectinase, protease, and β -glucosidase activities. Almost half of the isolates from Erub Island did not grow well at low temperature (scores lower than 3), whereas 96% of the Tasmanian isolates had a score equal to 4 (Table S4).

Screening under fermentation conditions

Based on the phenotyping results, 74 isolates (49 from Tasmania and 25 from Erub Island; Table 1), were selected and evaluated for their ability to ferment media containing glucose, fructose and maltose (GFM media). Metabolite concentrations were converted to discrete scores to visualise differences between isolates clearly. The ability to consume different sugars, the increased production of most metabolites and decreased production of acetic acid were considered positive attributes. Scores for all selected isolates are shown in Table S5. Hierarchical clustering revealed three main clusters (Figure 2).

Cluster 1 included isolates from Tasmania and Erub Island that efficiently utilised glucose and fructose, while producing malic acid and ethanol. Several of these isolates also produced various organic acids and glycerol.

Clusters 2 and 3 incorporated strains that used less sugar, however all *A. pullulans* isolates, and one *C. intermedia* strain were the only isolates capable of utilising maltose in the presence of glucose and fructose (Figure 2).

Beer, cider and wine chemical composition

Eight isolates were selected to evaluate their volatile profile during the fermentation of wort, apple juice and grape juice. One isolate from each of the main species screened under fermentation conditions and which showed high scores for stress tolerance, enzyme activities and/or fermentation performance was selected. These isolates included *Debaryomyces*_P1_B5, *H. osmophila*_P3_A12, *H. valbyensis*_P2_C3, *L. cidri*_P2_E4, and *R. mucilaginosa*_P3_B7 from Tasmania, and *A. pullulans*_P3_C1, *C. stellimalicola*_P3_E8, and *C. xylopsi*_P3_H4 from Erub Island. All fermentations were sequentially inoculated halfway through fermentation with a *S. cerevisiae* control strain.

Several differences in chemical composition were found in beers produced with the different yeast strains. Beers produced with *A. pullulans*, *C. stellimalicola* and *C. xylopsoci* all contained residual sugar (Table 2), with *A. pullulans* not effectively utilising maltose, maltotriose and glucose, *C. stellimalicola* maltose and maltotriose and *C. xylopsoci* maltose, maltotriose and fructose (Table S6). Minor differences in malic acid concentration were found across the treatments, while *A. pullulans* beers had the highest lactic acid concentration. Beers produced with *A. pullulans* and *C. stellimalicola* had the lowest glycerol concentrations. All beers had similar ethanol concentrations ranging from 3.4 – 4.0 % v/v (Table 2).

No significant differences were found for residual sugar in cider (Table S6). Ciders produced with *R. mucilaginosa* and ciders fermented with the control *S. cerevisiae* had the highest citric acid concentration, while ciders produced with *Candida* and *Hanseniaspora* species had the highest succinic acid concentration (Table 2). Minor differences were found for malic acid concentration among strains. *L. cidri* produced the highest lactic acid concentration, which was more than twice the amount produced by *S. cerevisiae*. No significant differences were found for glycerol or ethanol among treatments. Ethanol ranged from 4.8 – 5.7 % (v/v).

Similarly to cider, no significant differences were found for residual sugar in wine. Minor differences were found for citric and succinic acids among strains (Table 2). Wines produced with *A. pullulans*, *R. mucilaginosa* and *S. cerevisiae* had the highest malic acid concentration. *L. cidri* produced the highest lactic acid concentration, while *Candida* species produced the lowest. Wines produced with *C. xylopsoci* and *L. cidri* had the highest glycerol concentration. No significant differences were found for ethanol concentration among treatments, which ranged from 13.3 – 14.8 % (v/v).

Beer, cider and wine volatile profile

Two strategies were used to characterise the volatile profile of beers, ciders and wines produced with the different non-*Saccharomyces* isolates. First, the concentration of the main

esters, higher alcohols and volatile acids was determined, and second, non-targeted volatile fingerprinting was performed to identify other volatile metabolites responsible for the aroma and flavour of these fermented beverages.

In beer, *C. xylopsoci* and *C. stellimalicola* produced ethyl acetate at concentrations, 50- and 10-times higher than the *S. cerevisiae* control, respectively (Table S7). The total concentration of esters (excluding ethyl acetate) was also highest in beers produced with *C. xylopsoci* (Figure 3), primarily due to high concentrations of ethyl propanoate and 3-methylbutyl acetate (Table S7). In contrast, beers produced with *A. pullulans* and *R. mucilaginosa* had the lowest total concentration of esters. The concentration of higher alcohols was highest when fermenting with *A. pullulans* and *C. stellimalicola* due to increased concentrations of 2-methyl propanol and 3-methyl butanol. Acetic acid concentration was the highest in beers fermented with *C. stellimalicola* and *S. cerevisiae*, while *A. pullulans* beers had the lowest levels (Figure 3). The total concentration of volatile acids (excluding acetic acid) was highest in *C. xylopsoci* beers due to increased concentrations of 2-methyl propanoic, and 2- and 3-methyl butanoic acids.

High ethyl acetate concentrations were found in ciders produced with *C. xylopsoci*, *H. osmophila* and *H. valbyensis*. High total concentrations of esters were found in ciders using *A. pullulans*, *Debaryomyces* and *H. valbyensis* (Figure 3). While ciders produced with *A. pullulans* and *Debaryomyces* displayed increased concentrations of ethyl octanoate and ethyl decanoate, *H. valbyensis* ciders had increased concentration of 3-methylbutyl acetate (Table S7). Increased higher alcohol concentrations were found in ciders fermented with *C. xylopsoci*, *L. cidri* and *S. cerevisiae*. Whereas *C. xylopsoci* ciders had high 2-methyl propanol and 2- and 3-methyl butanol concentrations, *L. cidri* and *S. cerevisiae* ciders had increased 3-methyl butanol concentrations. Ciders produced with *C. stellimalicola*, *C. xylopsoci* and *H. valbyensis* showed the highest acetic acid concentrations, whereas *C. xylopsoci* and *L. cidri* had the highest concentration of volatile acids (Figure 3). Similarly to the results observed in beer, *C.*

xylopsoci produced increased concentrations of 2-methyl propanoic, and 2- and 3-methyl butanoic acids.

In wine, *C. xylopsoci*, *C. stellimalicola*, *L. cidri* and *H. osmophila* produced high ethyl acetate concentrations (Figure 3). However, these species showed different responses regarding total esters. Wines produced with *C. xylopsoci* and *C. stellimalicola* had increased concentrations of total esters, mainly due to 3-methylbutyl acetate, whereas *L. cidri* and *H. osmophila* wines had low total ester concentrations and reduced concentrations of most ethyl esters (Table S7). *L. cidri* wines had the highest concentration of higher alcohols due to 2-methyl propanol and 3-methyl butanol, while *H. osmophila* wines had the lowest. Acetic acid concentration was the highest in wines produced with *C. xylopsoci* and *H. osmophila* followed by *C. stellimalicola* wines. Wines fermented with *C. stellimalicola* also had the highest concentration of volatile acids including increased concentrations of 2-methyl propanoic, hexanoic, octanoic and decanoic acids (Table S7). *H. osmophila* wines had the lowest concentration of volatile acids.

Non-targeted volatile fingerprinting results were then used for principal component analysis (PCA) revealing numerous differences between yeast strains. Several clusters were observed for beer, with *C. stellimalicola*, *H. osmophila*, *H. valbyensis*, *L. cidri* and *S. cerevisiae* beers grouping together to the right of the PCA plot (Figure 4). This group was associated with 2- and 3-methyl butanol, 2,4-di-tert-butylphenol, some esters and several unknown volatile metabolites. *A. pullulans* beers grouped to the bottom of the plot and were associated to 2-methyl propanol and several unknown metabolites. In contrast, *C. xylopsoci* beers clustered at the top of the PCA plot and associated with ethyl propanoate and some unknown compounds. *R. mucilaginosa* beers grouped to the left of the first cluster, whereas beers produced with *Debaryomyces* clustered to the left of the PCA plot. These beers associated to ethyl-2-methyl propanoate, ethyl-2-methyl butanoate, ethyl-3-methyl butanoate and 2-phenylethyl acetate.

Several clusters were also observed for cider. *A. pullulans* ciders grouped to the right of the PCA plot and were related to 2-furanmethanol and several unknown volatile compounds (Figure 4). Ciders produced with *C. stellimalicola* and *H. valbyensis* grouped to the left of *A. pullulans* and associated to hexyl acetate and some unknown metabolites. Ciders produced with *Debaryomyces*, *R. mucilaginosa* and *S. cerevisiae* grouped at the bottom of the PCA plot and were related to ethyl octanoate, ethyl decanoate and some unknown metabolites. *H. osmophila* ciders grouped at the upper-left quadrant of the PCA plot and were associated to hexanol, propanol, ethyl-2-methyl butanoate and 3-methylbutyl acetate among others. The last cluster included cider fermented with *C. xylopsoci* and *L. cidri* which associate to 3-methyl butanol, 2,4-di-tert-butylphenol, benzaldehyde, ethyl nonanoate and several unknown volatile compounds.

In wine, a cluster containing *A. pullulans*, *Debaryomyces*, *H. valbyensis*, *R. mucilaginosa* and *S. cerevisiae* wines located in the upper-right quadrant of the PCA plot and associated to esters of medium chain fatty acids, including methyl -octanoate and -decanoate, and ethyl -hexanoate, -octanoate and -decanoate (Figure 4). A second group containing *C. stellimalicola* and *C. xylopsoci* wines located in the lower-right quadrant and was associated to geranyl acetate, hex-3-enyl acetate and 3-methylpentyl acetate. Wines produced with *H. osmophila* clustered in the lower-left quadrant and were related to 2-methylbutyl acetate, phenylethyl acetate and an unknown metabolite. *L. cidri* wines located in the upper-left quadrant of the plot and were associated with several higher alcohols and numerous unknown volatile compounds.

DISCUSSION

Numerous indigenous fermented beverages have been produced worldwide (Steinkraus, 1995). In Australia, *way-a-linah*, a mildly alcoholic beverage produced from the sap of the cider gum *E. gunnii*, and *tuba*, a fermented drink made from the syrup of the coconut tree *C. nucifera*, are two of several fermented beverages produced by Australia's Indigenous peoples

(Brady, 2008; Brady and McGrath, 2010). Here, we describe the characterisation of yeast isolates associated with the fermentation substrates of these two beverages and their potential to produce beer, cider and wine with diverse volatile profiles.

The native microbial communities associated with the fermentation of *E. gunnii* sap have only been reported recently (Varela et al., 2020). Whereas more than 130 fungal genera were detected using metagenomic approaches, only 14 different genera were identified among all the single isolates obtained from Tasmania. The most abundant genera reported by Varela et al. (2020) included *Kregervanrija*, *Hanseniaspora* and *Lachancea*, while in this study the most abundant genera among single isolates were *Hanseniaspora* and *Lachancea*. As isolates of *Kregervanrija* were not obtained in this study, it is very likely that the conditions used did not favour the growth of *Kregervanrija* species. In fact, it is also likely that other genera or species were also affected by the conditions employed for yeast isolation. Careful formulation of laboratory culture media mimicking the sample environment and thorough evaluation of culture conditions have maximised yeast diversity and isolation of uncultured novel species (Spurley et al., 2022; Thrash, 2019), and may help with isolating additional species associated with *E. gunnii*.

Since collection of sap from palm trees and *tuba* production appear no longer to be practiced in Australia, it was not possible to sample fermenting coconut palm syrup. Instead, we collected yeasts from palm tree inflorescences and coconuts but also from other flowers, fruits and plants, including hibiscus and frangipani flowers, bananas, paw paws and other native fruits in the vicinity. Numerous species have been described in association with flowers, plants, blossoms, and fruits, with *Aureobasidium*, *Candida*, *Meyerozyma*, *Metschnikowia*, *Hanseniaspora* and *Pichia* species being the most frequently isolated species (do Vale et al., 2021; Felix et al., 2021; Vadkertiová et al., 2012). While some of these are cosmopolitan, being globally distributed and associated with multiple substrates, including plant material, soil, and lacustrine and marine environments, others are found only in limited environmental

niches (Felix et al., 2021). Some of these species were also found in samples from Erub Island with *Aureobasidium* and *Candida* species being the most abundant.

Yeast isolates differed substantially between the two sampling locations studied in this work. This is likely the result of multiple factors such as substrate of isolation, altitude, temperature, and climate. While the Central Plateau in Tasmania has a high altitude (1000 m above sea level), cooler temperatures (< 20 °C year-round) and frosts over winter, Erub Island is a volcanic island with a maximum altitude of 180 m, tropical climate, higher temperatures (27 °C average) and high humidity. Interestingly, nearly half of the isolates from Erub Island did not grow well at 12 °C suggesting that they are adapted to moderate temperatures. In fact, several environmental and anthropogenic factors shape the abundance and/or the composition of the microbial communities found in soil or associated with plants and fruits (Alsammar et al., 2019; Burns et al., 2015; Gleeson et al., 2016; Lin et al., 2018; Montecchia et al., 2015; Siles and Margesin, 2016).

Screening for tolerance to stress conditions, enzyme activities and sugar utilisation enabled grouping of isolates based on their phenotype and visualisation of patterns. Strains from the same species predominantly grouped together, whereas some clustering according to location was also observed. While glucose, fructose, and maltose are the main sugars in *E. gunnii* sap (Varela et al., 2020) and honey (Steinkraus, 1995), sucrose, glucose and fructose are the most abundant sugars in coconut sap (Purnomo, 1992) and maple syrup (Stuckel and Low, 1996). Most of the isolates evaluated in a medium containing different sugars, efficiently consumed glucose and fructose but not maltose. This indicates that carbon repression mechanisms inhibit the utilisation of maltose in the presence of preferred carbon sources (Gancedo, 1998), potentially restricting the use of these isolates as a single inoculum for beer fermentation.

Non-*Saccharomyces* species are generally not capable of completing alcoholic fermentation unaided and therefore co-inoculation or sequential inoculation strategies with *S. cerevisiae*

are needed (Ciani et al., 2006; Jolly et al., 2006; Soden et al., 2000). A key consideration for these strategies is the compatibility between the non-*Saccharomyces* yeast used and *S. cerevisiae*. Negative interactions have been described for wine and beer production (Harlé et al., 2020; Holt et al., 2018). In fact, different *S. cerevisiae* strains respond differently to distinct non-*Saccharomyces* species, affecting yeast fitness and fermentation performance (Onetto et al., 2021). Since different control *S. cerevisiae* strains were used for wort, apple and grape juices, these reports may explain why some fermentations were completed in some matrices but not others; for example fermentations with *A. pullulans* were only completed in grape juice, while *C. xylopsi* fermentations were completed only in apple juice. Using different *S. cerevisiae* strains in these cases may ensure completion of fermentation.

The use of non-*Saccharomyces* species is seen as a promising strategy to improve and differentiate the organoleptic profile of fermented beverages, in fact several non-*Saccharomyces* yeast species have been assessed for their ability to produce beer, cider, and/or wine (Bourbon-Melo et al., 2021; Gamero et al., 2020; Holt et al., 2018; Lorenzini et al., 2019; Wei et al., 2020). Overall, the species evaluated here showed diverse volatile profiles compared to the *S. cerevisiae* controls indicating the potential of these species to produce distinctive fermented beverages. However, a different volatile profile does not necessarily mean a 'better' sensory profile. Several studies have reported that while some non-*Saccharomyces* yeast strains provide an improved overall aroma and palate, some do the opposite (Bourbon-Melo et al., 2021; Dashko et al., 2015; Wei et al., 2019; Wei et al., 2020). While volatile composition provides some indication of strain propensity to generate a desirable organoleptic profile, sensory evaluation is the only way to confirm it. Linking volatile composition with undesirable sensory profiles is sometimes more straightforward, for instance, the high concentrations of ethyl acetate produced by *C. xylopsi* in beer, cider, and wine, would most likely result in a negative sensory profile. While at low levels (approximately 50 mg/L) ethyl acetate may add aroma complexity, at concentrations above 150 mg/L it is associated with negative sensory descriptors, such as nail polish remover (Amerine and

Roessler, 1983; Jackson, 2009). Since several unknown volatile metabolites were responsible for discrimination between yeast treatments, it is key to identify them and determine their contribution to the aroma and flavour of fermented beverages. Future research should also evaluate the sensory profile of the resulting beverages.

In conclusion, non-*Saccharomyces* species associated with fermentations historically conducted by Australia's Indigenous peoples, were used to produce beer, cider, and wine with diverse volatile profiles. Thus, this study is the first step for the utilisation of native yeast species in the production of fermented beverages with distinctive aroma and flavour. In addition, our findings highlight the enormous potential of yeasts associated with native Australian plants and beverages.

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AUTHOR CONTRIBUTIONS

CV collected samples, analysed samples and results, drafted the manuscript and participated in the conception of the work. LA processed samples, characterised isolates and performed fermentations. JS collected and processed samples. MS performed chemical analyses. MB participated in the conception of the work. AB analysed results and participated in the conception of the work. VJ collected samples, participated in the conception of the work and secured funding. All authors reviewed the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. Microbial isolates were obtained from two different geographical locations in Australia, the Central Plateau in Tasmania, and Erub Island in the Torres Strait, approximately 3,600 km apart. In Tasmania, bark, sap and soil samples were collected from the cider gum *Eucalyptus gunnii*, while in Erub Island samples were collected from flowers, fruits and plants.

Figure 2. Hierarchical clustering of selected isolates. Isolates were screened for: their tolerance to common stress conditions found during the production of fermented beverages; enzyme activities relevant to the appearance, aroma and flavour of these beverages; and their ability to ferment chemically defined GFM media. Colony sizes, enzyme activities and metabolite concentrations were converted to discrete scores to visualise differences between isolates clearly. The ability to grow under different stress conditions, the presence of enzyme activities, low H₂S production, the ability to consume different sugars, the production of most metabolites and low acetic acid production were considered positive attributes and had high score values. Isolates selected for fermentation of wort, apple juice and grape juice are indicated in red.

Figure 3. Heatmap indicating the concentration of esters, higher alcohols and volatile acids in beer, cider and wine sequentially fermented with different non-*Saccharomyces* isolates and *S. cerevisiae*. Ethyl esters, acetate esters and total ester concentration excludes ethyl acetate, total concentration of volatile acids excludes acetic acid. Different *S. cerevisiae* control strains (SC) were used for the different beverages, Safale US-05 for beer, Safcider AB1 for cider and Lalvin EC1118 for wine. All ferments were sequentially inoculated with *S. cerevisiae* controls halfway through fermentation.

Figure 4. Principal component analysis for non-targeted volatile analysis of beer, cider and wine sequentially fermented with different non-*Saccharomyces* isolates and *S. cerevisiae*.

799 AuPul *A. pullulans*; CanSte, *C. stellimalicola*; CanXyl, *C. xylopsoci*; Deb, *Debaryomyces spp*;
800 HanValb, *H. valbyensis*; HanOsm, *H. osmophila*; LaCidri, *L. cidri*; RhoMu, *R. mucilaginosa*.
801 Different *S. cerevisie* control strains (SC) were used for the different beverages, Safale US-
802 05 for beer, Safcider AB1 for cider and Lalvin EC1118 for wine. All ferments were sequentially
803 inoculated with *S. cerevisae* controls halfway through fermentation.

804

805 **Figure S1.** Hierarchical clustering of all isolates. Isolates were screened for: their tolerance to
806 common stress conditions found during the production of fermented beverages and enzyme
807 activities relevant to the appearance, aroma and flavour of these beverages. Colony sizes and
808 enzyme activities were converted to discrete scores to visualise differences between isolates
809 clearly. The ability to grow under different stress conditions, the presence of enzyme activities
810 and low H₂S production were considered positive attributes and had high score values.
811 Isolates selected for fermentation screening are indicated in red.

Table 1. Number of isolates identified in samples collected from Tasmania and Erub Island and selected for colony phenotyping and fermentation screening.

Species	Total number	Isolates for colony phenotyping	Isolates for fermentation screening
Tasmania			
<i>Aureobasidium sp.</i>	2*	-	-
<i>Candida railenensis</i>	3*	1	1
<i>Debaryomyces sp.</i>	13*	6	6
<i>Hanseniaspora sp.</i>	2*	1	-
<i>Hanseniaspora osmophila</i>	91	39	10
<i>Hanseniaspora valbyensis</i>	767	57	12
<i>Hypoxyton sp.</i>	5	5	5
<i>Lachancea cidri</i>	138	29	9
<i>Malassezia sp.</i>	1*	-	-
<i>Metschnikowia sp.</i>	1*	-	-
<i>Paraphaeosphaeria michotii</i>	1	1	1
<i>Penicillium citreonigrum</i>	1	1	1
<i>Rhodotorula sp.</i>	4	4	4
Total	1029	144	49
Erub Island			
<i>Aureobasidium pullulans</i>	6	6	6
<i>Candida sp.</i>	14*	13	5
<i>Candida intermedia</i>	8	8	3
<i>Candida stellimalicola</i>	21*	12	4
<i>Candida tallmaniae</i>	5	5	2
<i>Candida xylopsoci</i>	2	2	2
<i>Hanseniaspora sp.</i>	2	2	1
<i>Metschnikowia sp.</i>	1*	-	-
<i>Meyerozyma guilliermondii</i>	1	1	1
<i>Penicillium sp.</i>	8*	-	-
<i>Phoma multirostrata</i>	2*	-	-
<i>Pichia sp.</i>	1	1	1
Total	71	50	25

815 * some isolates from these species did not grow after freeze-thawing and could not be
816 phenotyped

817 **Table 2.** Summary of the main chemical compositional differences for beer, cider and wine produced with different non-*Saccharomyces* yeast
818 strains.

	Sugar [g/L]	Citric acid [g/L]	Malic acid [g/L]	Succinic acid [g/L]	Lactic acid [g/L]	Glycerol [g/L]	Ethanol [% v/v]
Wort	61.8						
<i>A. pullulans</i>	5.2 ± 0.3 ^a	0.3 ± 0.0 ^a	0.2 ± 0.0 ^{ab}	0.2 ± 0.1 ^a	3.5 ± 0.2 ^a	1.5 ± 0.2 ^c	4.0 ± 0.1 ^a
<i>C. stellimalicola</i>	2.8 ± 0.4 ^{bc}	0.3 ± 0.0 ^a	0.4 ± 0.1 ^a	0.3 ± 0.0 ^a	2.2 ± 0.2 ^{bc}	1.5 ± 0.3 ^c	3.6 ± 0.1 ^{ab}
<i>C. xylopsoci</i>	3.2 ± 0.1 ^b	0.3 ± 0.0 ^a	0.2 ± 0.1 ^b	0.0 ± 0.0 ^a	1.6 ± 0.8 ^c	2.8 ± 0.3 ^a	3.4 ± 0.2 ^b
<i>Debaryomyces sp.</i>	0.2 ± 0.3 ^d	0.3 ± 0.0 ^a	0.3 ± 0.0 ^{ab}	0.0 ± 0.0 ^a	2.0 ± 0.3 ^{bc}	2.4 ± 0.1 ^{ab}	3.6 ± 0.1 ^{ab}
<i>H. osmophila</i>	0.0 ± 0.0 ^d	0.3 ± 0.0 ^a	0.3 ± 0.0 ^{ab}	0.1 ± 0.1 ^a	1.6 ± 0.2 ^c	2.2 ± 0.1 ^b	3.7 ± 0.1 ^{ab}
<i>H. valbyensis</i>	0.0 ± 0.0 ^d	0.3 ± 0.0 ^a	0.2 ± 0.0 ^{ab}	0.1 ± 0.2 ^a	1.2 ± 0.5 ^c	2.3 ± 0.1 ^b	3.6 ± 0.2 ^{ab}
<i>L. cidri</i>	0.0 ± 0.0 ^d	0.3 ± 0.0 ^a	0.2 ± 0.0 ^{ab}	0.0 ± 0.0 ^a	1.9 ± 0.1 ^c	2.4 ± 0.0 ^{ab}	3.7 ± 0.0 ^{ab}
<i>R. mucilaginosa</i>	1.1 ± 1.8 ^{cd}	0.3 ± 0.0 ^a	0.2 ± 0.0 ^{ab}	0.1 ± 0.1 ^a	3.1 ± 0.4 ^{ab}	2.7 ± 0.1 ^{ab}	3.5 ± 0.1 ^b
<i>S. cerevisiae</i>	0.0 ± 0.0 ^d	0.3 ± 0.1 ^a	0.2 ± 0.1 ^{ab}	0.2 ± 0.1 ^a	1.4 ± 0.5 ^c	2.4 ± 0.3 ^{ab}	3.5 ± 0.2 ^b
Apple juice	96.9						
<i>A. pullulans</i>	15.2 ± 25.5 ^a	1.3 ± 0.2 ^{cde}	5.4 ± 0.3 ^a	0.5 ± 0.1 ^{bc}	1.4 ± 0.2 ^c	4.4 ± 1.3 ^a	4.8 ± 1.5 ^a
<i>C. stellimalicola</i>	9.0 ± 5.2 ^a	1.4 ± 0.2 ^{bcd}	4.9 ± 0.4 ^{ab}	1.1 ± 0.1 ^a	2.4 ± 0.2 ^{bc}	4.2 ± 0.1 ^a	5.1 ± 0.4 ^a
<i>C. xylopsoci</i>	0.5 ± 0.1 ^a	0.7 ± 0.0 ^{ef}	5.0 ± 0.2 ^{ab}	0.8 ± 0.1 ^{ab}	2.5 ± 1.0 ^{bc}	5.1 ± 0.2 ^a	5.7 ± 0.0 ^a
<i>Debaryomyces sp.</i>	0.5 ± 0.1 ^a	1.8 ± 0.3 ^{bc}	4.9 ± 0.0 ^{ab}	0.5 ± 0.1 ^{bc}	2.1 ± 0.7 ^{bc}	3.9 ± 0.8 ^a	5.7 ± 0.1 ^a
<i>H. osmophila</i>	0.4 ± 0.0 ^a	1.1 ± 0.2 ^{def}	5.0 ± 0.4 ^{ab}	1.1 ± 0.2 ^a	3.7 ± 0.5 ^{ab}	4.3 ± 0.3 ^a	5.5 ± 0.4 ^a
<i>H. valbyensis</i>	0.4 ± 0.1 ^a	0.5 ± 0.1 ^f	5.2 ± 0.1 ^{ab}	1.1 ± 0.1 ^a	2.1 ± 0.6 ^{bc}	5.1 ± 0.3 ^a	5.6 ± 0.1 ^a
<i>L. cidri</i>	0.4 ± 0.1 ^a	0.6 ± 0.0 ^f	4.7 ± 0.4 ^{ab}	0.6 ± 0.1 ^b	5.0 ± 0.6 ^a	3.9 ± 0.6 ^a	5.6 ± 0.1 ^a
<i>R. mucilaginosa</i>	0.4 ± 0.0 ^a	2.1 ± 0.2 ^{ab}	4.5 ± 0.1 ^b	0.5 ± 0.2 ^{bc}	2.0 ± 1.2 ^{bc}	4.1 ± 0.6 ^a	5.7 ± 0.1 ^a

<i>S. cerevisiae</i>	0.5 ± 0.3 ^a	2.6 ± 0.4 ^a	4.5 ± 0.5 ^b	0.2 ± 0.1 ^c	2.1 ± 0.5 ^{bc}	4.9 ± 0.5 ^a	5.6 ± 0.1 ^a
Grape juice	232.7						
<i>A. pullulans</i>	0.2 ± 0.2 ^a	0.6 ± 0.1 ^a	3.5 ± 0.1 ^a	0.8 ± 0.1 ^b	2.4 ± 0.1 ^{cd}	8.1 ± 0.2 ^{bcd}	14.8 ± 0.0 ^a
<i>C. stellimalicola</i>	0.3 ± 0.1 ^a	0.3 ± 0.0 ^b	3.0 ± 0.1 ^{bc}	0.5 ± 0.1 ^c	1.8 ± 0.1 ^e	7.3 ± 0.2 ^e	14.7 ± 0.0 ^a
<i>C. xylopsoci</i>	18.9 ± 24.2 ^a	0.4 ± 0.0 ^b	3.0 ± 0.3 ^{bc}	0.4 ± 0.1 ^c	1.2 ± 0.1 ^f	9.7 ± 0.5 ^a	13.3 ± 1.9 ^a
<i>Debaryomyces sp.</i>	0.4 ± 0.3 ^a	0.6 ± 0.0 ^a	3.4 ± 0.2 ^{ab}	0.9 ± 0.1 ^{ab}	2.3 ± 0.3 ^d	8.0 ± 0.2 ^{cd}	14.7 ± 0.0 ^a
<i>H. osmophila</i>	5.6 ± 4.1 ^a	0.4 ± 0.1 ^b	2.5 ± 0.1 ^d	1.1 ± 0.1 ^b	3.0 ± 0.2 ^{ab}	7.5 ± 0.3 ^{de}	14.5 ± 0.2 ^a
<i>H. valbyensis</i>	0.4 ± 0.3 ^a	0.6 ± 0.0 ^a	3.4 ± 0.1 ^a	0.9 ± 0.0 ^{ab}	2.2 ± 0.2 ^d	8.1 ± 0.1 ^{cd}	14.7 ± 0.1 ^a
<i>L. cidri</i>	0.1 ± 0.1 ^a	0.3 ± 0.0 ^b	2.7 ± 0.0 ^{cd}	1.1 ± 0.2 ^{ab}	3.4 ± 0.1 ^a	9.6 ± 0.1 ^a	14.4 ± 0.1 ^a
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819

820 Letters indicate statistically significant differences between yeast strains.

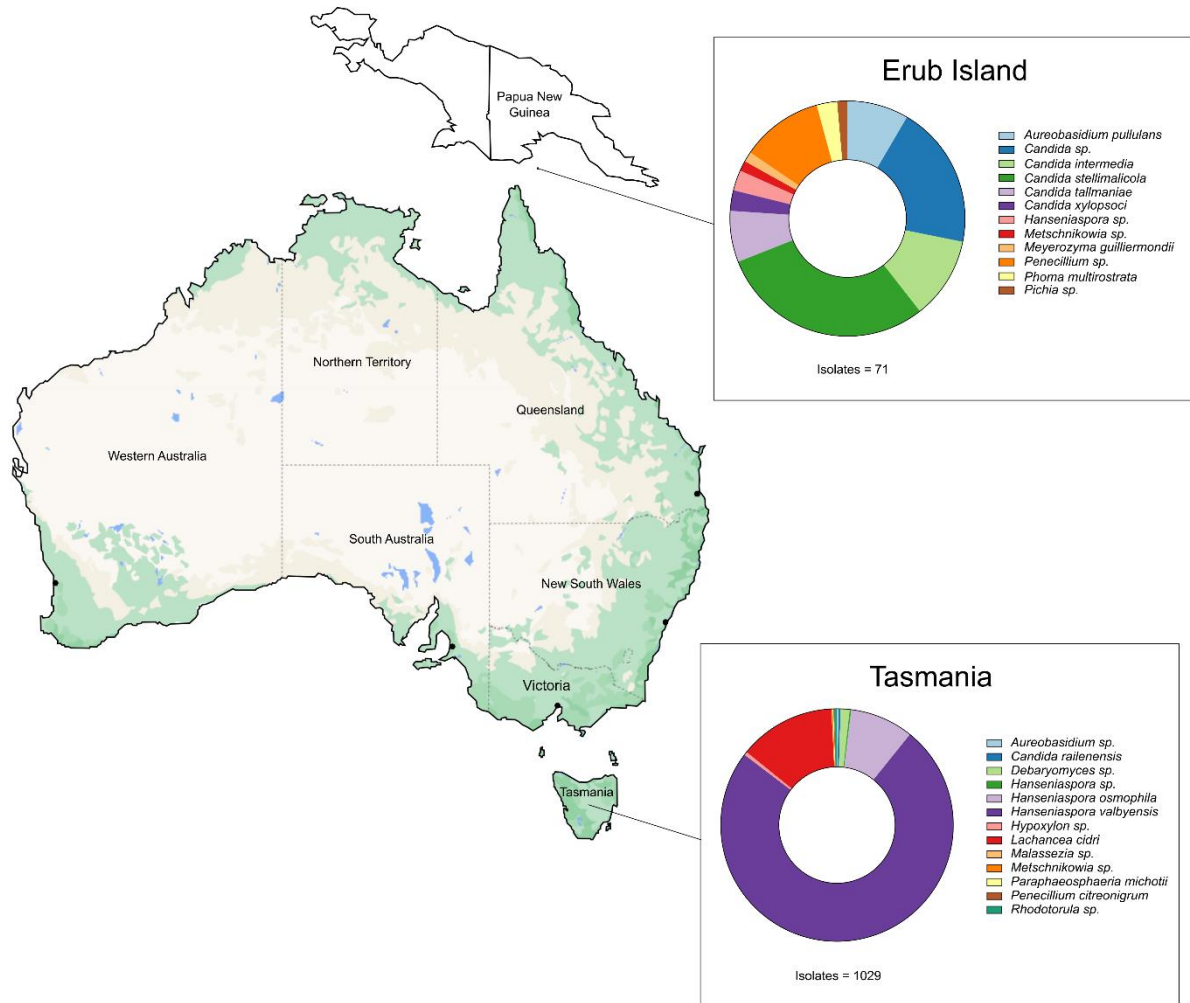


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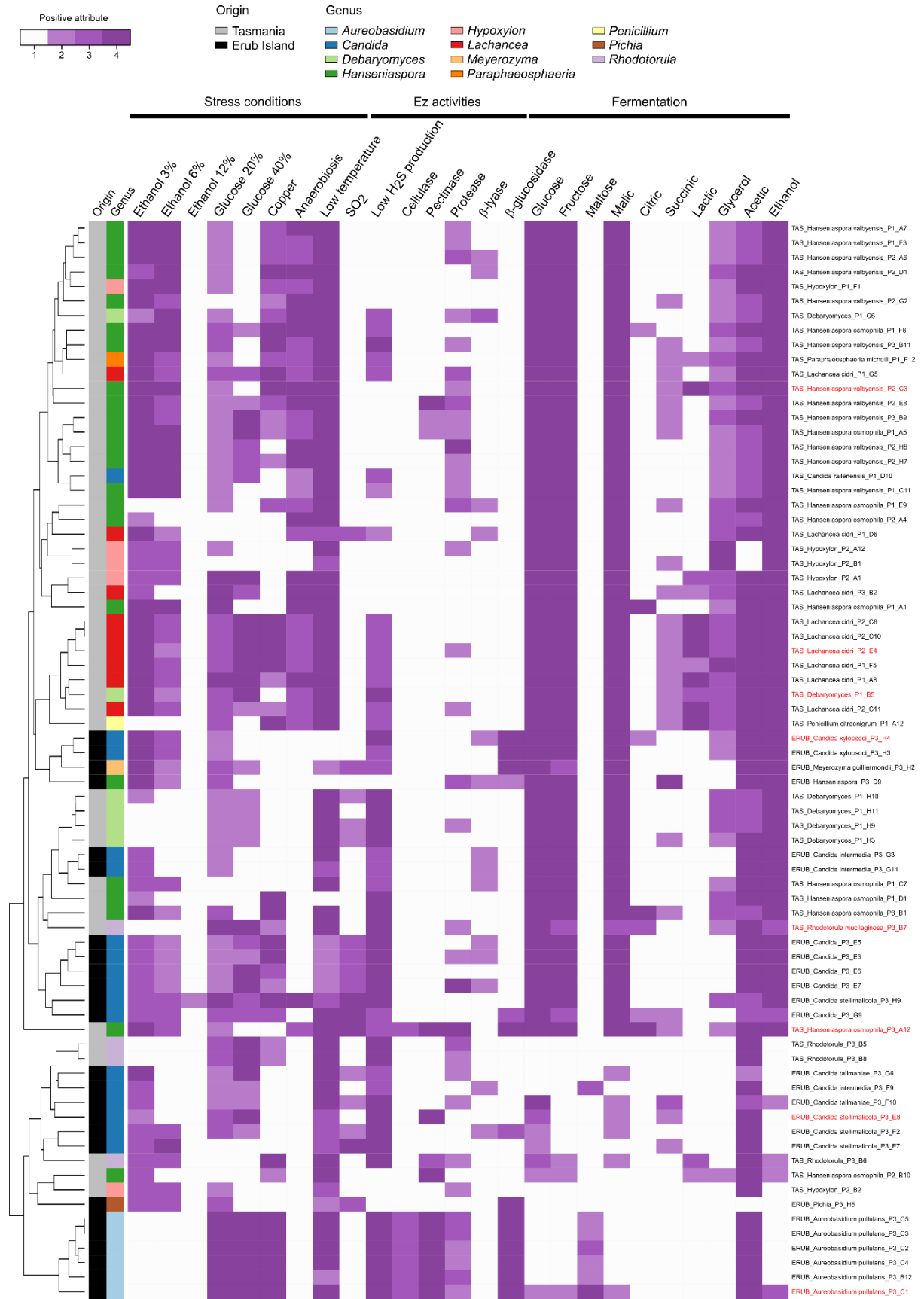


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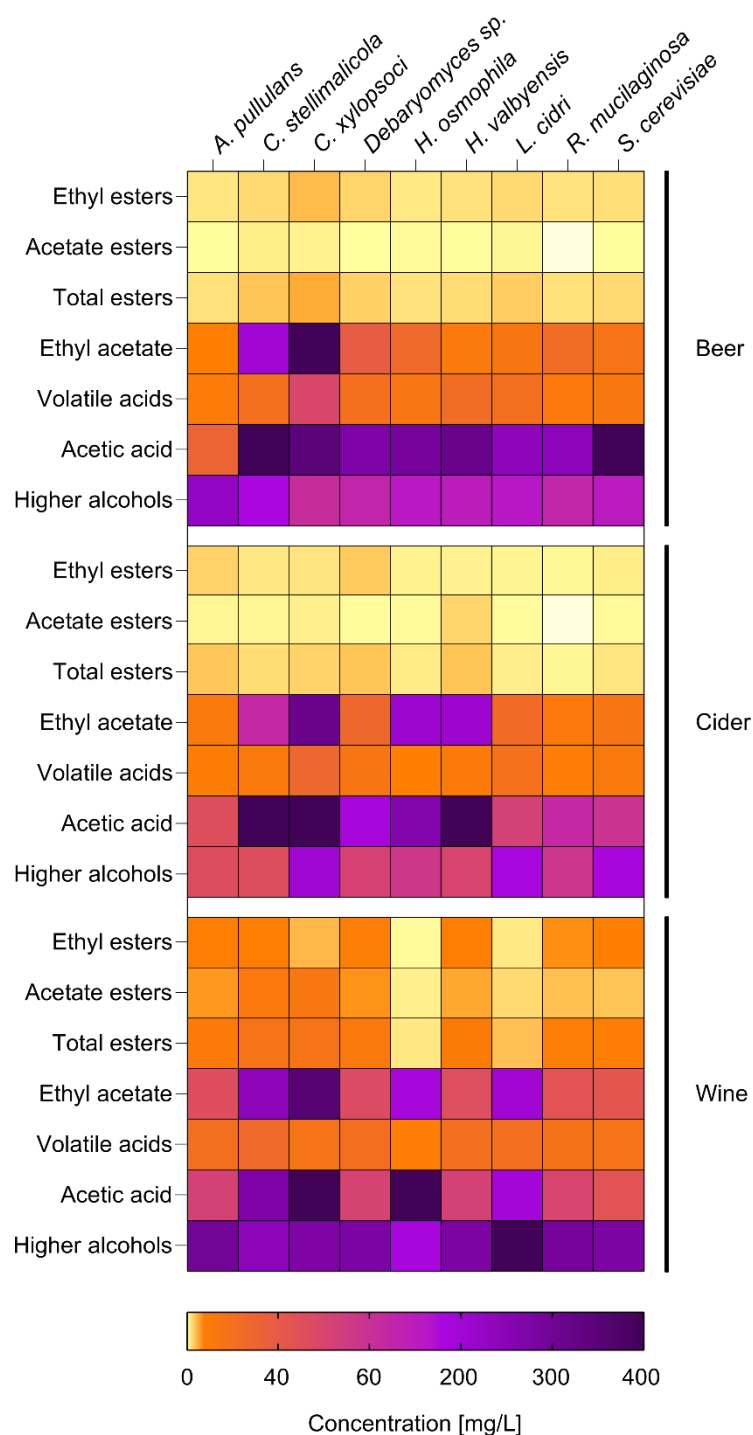


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