New yeasts - new brews: modern approaches to brewing yeast design and development

Gibson, B.[1*], Geertman, J.-M.A.[2], Hittinger, C.T.[3], Krogerus, K.[1,4], Libkind, D.[5], Louis, E.J.[6], Magalhães, F.[1,4], Sampaio, J.P.[7]

[1] VTT Technical Research Centre of Finland Ltd, Tietotie 2, P.O. Box 1000, FI-02044 VTT, Espoo, Finland

[2]Heineken Supply Chain, Global Research & Development, 2382 PH Zoeterwoude, The Netherlands

[3]Laboratory of Genetics, Genome Center of Wisconsin, DOE Great Lakes Bioenergy Research Center, Wisconsin Energy Institute, J. F. Crow Institute for the Study of Evolution, University of Wisconsin-Madison, Madison, WI 53705, USA

[4]Department of Biotechnology and Chemical Technology, Aalto University, School of Chemical Technology, Kemistintie 1, Aalto, P.O. Box 16100, FI-00076 Espoo, Finland

[5]Laboratorio de Microbiología Aplicada, Biotecnología y Bioinformática de Levaduras, Instituto Andino Patagónico de Tecnologías Biológicas y Geoambientales, IPATEC (CONICET-UNComahue), Centro Regional Universitario Bariloche, Bariloche, Río Negro, Argentina

[6]Centre for Genetic Architecture of Complex Traits, Dept. of Genetics, University of Leicester, University Road, Leicester LE1 7RH, UK

[7]UCIBIO-REQUIMTE, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

*corresponding author

Abstract

The brewing industry is experiencing a period of change and experimentation largely driven by customer demand for product diversity. This has coincided with a greater appreciation of the role of yeast in determining the character of beer and the widespread availability of powerful tools for yeast research. Genome analysis in particular has helped clarify the processes leading to domestication of brewing yeast and has identified domestication signatures that may be exploited for further yeast development. The functional properties of non-conventional yeast (both *Saccharomyces* and non-*Saccharomyces*) are being

assessed with a view to creating beers with new flavours as well as producing flavoursome non-alcoholic beers. The discovery of the psychrotolerant *S. eubayanus* has stimulated research on *de novo S. cerevisiae* x *S. eubayanus* hybrids for low-temperature lager brewing and has led to renewed interest in the functional importance of hybrid organisms and the mechanisms that determine hybrid genome function and stability. The greater diversity of yeast that can be applied in brewing, along with an improved understanding of yeasts' evolutionary history and biology, is expected have a significant and direct impact on the brewing industry, with potential for improved brewing efficiency, product diversity and, above all, customer satisfaction.

Introduction

Saccharomyces cerevisiae may be considered the perfect model of a model organism. Its short replication time, simple cultivation, sporulation efficiency, rare pathogenicity, and small genome size (6000 genes) have made it an ideal research organism and placed it at the forefront of many scientific advances. The species has been used to study medicine (Mager & Winderickx, 2005), evolution (Voordeckers & Verstrepen, 2015), and population genomics (Liti et al., 2009). Engineered strains are also being used in the production of pharmaceuticals and other important chemicals (Borodina & Nielsen, 2014). The *S. cerevisiae curriculum vitae* includes an impressive list of firsts: first eukaryotic organism to have its genome sequenced (Goffeau et al., 1996), first genetically modified (GM) organism approved for a food application (Aldhous, 1990), and first synthetic eukaryotic chromosome (Annaluru et al., 2014). The species is also on its way to being the first eukaryote to have its genome recreated synthetically (Richardson et al., 2017).

In the midst of such credentials, it is easy to overlook the primary biotechnological function of *S. cerevisiae* and its close relatives: food and beverage production. Products resulting from yeast metabolic activity include not only bread and fermented beverages like beer and wine but also chocolate, coffee, and various other foods (Ardhana & Fleet, 2003; Avallone et al., 2001; Batista et al., 2015). These products would not exist, or would exist in an inferior form, without yeasts being involved in the production process. It must also be pointed out that *S. cerevisiae* would not enjoy its elevated status as a model organism if not for its primary role in food production. Pasteur and his contemporaries in the 19th century significantly advanced our understanding of microbiology, fermentation, and biochemistry through their studies of yeasts (Barnett & Lichtenthaler, 2001). However, the initial impetus for such research was more prosaic: how to prevent the spoilage of wine and beer (Pasteur, 1873; 1876). Indeed, for some time many fundamental scientific breakthroughs were made in the course of applied research on industrial yeasts. The brewing industry, in particular, was an early supporter and benefactor of such research. Important

advances in yeast taxonomy, biochemistry, and genetics, as well as in the development of practical techniques, such as preparation of single-cell cultures, were made in brewery laboratories (Barnett & Lichtenthaler, 2001). Given the brewing industry's enthusiasm for yeast research, it is perhaps surprising to note that most yeast strains currently used in the production of beer have not undergone any form of intentional development to improve their performance. Strains that possess superior properties likely acquired them in the preceding centuries as they were domesticated.

The brewing industry is a traditional one, and despite industrialization and modernization, the process of producing beer is not fundamentally different to that practised prior to industrialization. The retention of particular strains to produce particular beers is one aspect of this respect for traditional brewing practises. The industry has also had to be sensitive to the wishes of consumers, especially with regard to the use of genetically modified organisms (GMO). The public's scepticism regarding GMO use in food is complex and related to a number of factors, including mistrust of big business, fear of possible health implications, and perception of GMO as being unnatural (Lusk et al., 2014). Since beer is a natural product, many consumers feel less comfortable with the application of GM technology in brewing than in the production of other processed foods (Tenbült et al., 2008). Despite considerable optimism in the early years, and real potential for improvement of fermentation efficiency and product quality, GM yeasts have never been used in commercial brewing (Boulton, 2015), though they have been used in the wine industry in the US following approval (Volschenk et al., 2004).

However, in the age of accessible and affordable genome analysis, we now have a greater insight into brewing yeast biology and evolution than at any time in the past. New tools have provided us with an improved understanding of the biological processes occurring in brewing yeasts and during brewery fermentation, enabling researchers and developers to make improvements without compromising the 'natural' state of the yeasts. Such approaches include the selection of appropriate brewing strains; the use of alternative yeasts, including non-*Saccharomyces* yeasts; the generation of new intra- and interspecies hybrids; and harnessing the adaptability of genomes to maximise pre-existing traits. Such approaches are expected to offer real benefits to both the brewer and the customer in the future through improved resource efficiency and greater product diversity. We provide here an overview of recent brewing yeast developments and the technologies that have facilitated these advances.

Ale yeast genome analysis as a tool to aid selection and development

Ale and lager yeasts, also known as the top-fermenting and bottom-fermenting yeasts, respectively, are the two main types of brewing yeasts used. Ale yeasts give rise to diverse beers but, in spite of the differences of the final product, most ale-brewing strains belong to *S. cerevisiae*. Lager yeasts are assigned

to *S. pastorianus* and are allopolyploid hybrids of *S. cerevisiae* and *S. eubayanus* (Dunn and Sherlock 2008; Nakao et al., 2009; Libkind et al., 2011). Given that lager yeasts are responsible for more than 90% of the beer produced worldwide, much more attention has been given to them. Until recently, little was known about the phylogenetic relationships of the ale yeast strains used to ferment different types of beers, as well as their relationships with non-brewing strains of *S. cerevisiae*.

A first indication that wine and ale beer strains were genetically distinct was provided by microsatellite markers (Legras et al., 2007). Using complete genome sequences, Gallone *et al.* (2016) and Gonçalves et al., (2016) investigated a comprehensive collection of ale-type beer yeasts and showed that they were fundamentally distinct from other industrially relevant strains of *S. cerevisiae*. Ale-type strains were grouped in a main cluster that included various types of German, British, Belgian, and American beers. However beer strains were also found to cluster in the wine, bread, and sake clades, as well as in an independent clade sister to the wine clade. Those studies also showed that beer yeasts have a high incidence of polyploidy and aneuploidy and, probably as a consequence of this, limited or no sporulation ability. Genome analyses and large-scale phenotyping of industry-specific traits revealed domestication signatures of ale brewing yeasts. For example, ale-type strains show a significantly greater capacity to metabolise maltotriose (Gallone et al., 2016). Another characteristic that appears to have been selected during brewing yeast domestication is for reduced production of phenolic off flavors (POF). Population genomics showed the acquisition of distinct inactivating mutations through convergent evolution during domestication (Gallone et al., 2016; Gonçalves et al., 2016), giving rise to the POF-negative phenotype. Since, POFs are desired flavor components for some beer styles, such as the Bavarian wheat beers and some Belgian beers, strains that ferment these beer types have functional *PAD1* and *FDC1* genes, which represents the ancestral state seen in wild strains and other industrial lineages, such as wine yeasts (Gonçalves et al., 2016).

Marker-assisted breeding is a strategy of molecularly tracking genes known to control traits. The organisms themselves are not GM; instead, molecular methods are merely used to help breeders predict which progeny will have desired traits. Breeders can then limit the resources for detailed phenotypic characterizations to strains already known to contain desired genetic variants. This strategy is frequently used in crop and livestock breeding, and Gallone et al., (2016) recently demonstrated that it is possible to efficiently select superior segregants from intraspecific hybrids in large-scale yeast breeding schemes.

Alternative yeast for alternative beers

Saccharomyces eubayanus

S. eubayanus, the latest addition to the genus *Saccharomyces*, was originally found in South America, Argentina (Libkind et al., 2011). Since then, there have been a number of isolations elsewhere, including North America (Peris et al., 2014, 2016), East Asia (Bing et al., 2014), and New Zealand (Gayevskiy & Goddard 2016) but, interestingly, not yet from Europe. So far, five genetic populations have been detected, two in South America (Peris et al., 2014; 2016), and three in Asia, in Tibet, Sichuan, and western China (Bing et al., 2014), although the latter have enough genetic differences to be potentially considered a subspecies or a different variety. Interestingly, one population from the Tibetan plateau and a few strains from USA show the closest genetic similarity to the *S. eubayanu*s portion of *S. pastorianus* (with ca. 99.8 % sequence similarity based on comparative genomics) (Peris et al., 2016). However, no known extant strain seems to be the direct ancestor of lager-brewing yeasts. Given that no natural populations of *S. eubayanus* have been detected hitherto in Europe, it has been suggested that the non-*S. cerevisiae* sub-genome of lager yeast is of Asian origin (Bing et al., 2014). However, genomic studies have shed new light on this issue, suggesting that a primary dispersal from South America into the Holarctic may be more likely based on the relative diversities of the Holarctic and one of the two Patagonian subpopulations and the confinement of a signature of recent demographic expansion to the Tibetan subpopulation (Peris et al., 2016). A yet-undiscovered European population of *S. eubayanus* is likely to exist and was probably involved in the original hybridization event (or events) that gave rise to the lager yeast. Ongoing efforts to reveal *S. eubayanus* distribution and occurrence in Patagonia (Argentina and Chile) have yielded over 200 isolates sorted in at least 5 local lineages, and the region is characterized by the highest (by 10 fold) natural occurrence of this yeast when compared to other geographic areas (unpublished results).

The discovery of *S. eubayanus*, the non-*S. cerevisiae* parent of the lager-brewing yeasts, occurred at a time when there existed in the beer market a growing demand for innovative products and a need to deliver to the customer more complex, or at least different, beer flavours. Thus, almost immediately after the strains of *S. eubayanus* became available, studies aimed at elucidating their brewing potential were initiated. The brewing properties of *S. eubayanus* have been so far only studied in the type strain of the species. For example, Gibson et al. (2013) found it to outperform most lager strains when cultured at low temperatures (10°C) in 2% glucose or maltose laboratory media. Similarly, Walther et al. (2014) showed that, even in synthetic media at 20 °, *S. eubayanus* was still competitive with respect to growth rates when compared to several brewing strains. In line with the psychrotolerant nature of *S. eubayanus*, its performance in lab conditions significantly diminished when it was grown at temperatures ≥ 25 °C (Walther et al., 2014; Mertens et al., 2015). In brewing wort, regardless of the fermenting temperature, *S. eubayanus* performed poorly, producing less ethanol than lager strains (Gibson et al., 2013; Krogerus et al., 2015; Mertens et al., 2015).

Sugar uptake is one of the major bottlenecks limiting the application of *S. eubayanus* to beer production; specifically, it is unable to ferment maltotriose (Gibson et al., 2013; Krogerus et al., 2015). Maltotriose uptake is a common and desirable trait of most lager yeasts because it is one of the major carbon sources in wort (Hough et al., 1982). The origin of maltotriose transporters in *S. pastorianus* is, however, still not clear. *MAL11*/*AGT1* has been proposed to encode the transporter responsible for maltotriose uptake in *S. cerevisiae*, although in *S. pastorianus* these genes are not functional (Vidgren et al., 2005; Vidgren & Londesborough 2012; Cousseau et al., 2013). This observation lead to the belief that the *lager-AGT1* (*SbAGT1* or *SeAGT1* in some references) and *MTT1*/*MTY1* were transmitted to the hybrid genome by the psychrotolerant parent. Both genes have higher similarity to genes from *S. cerevisiae* than from the *S. eubayanus* type strain, although not high enough to allow for definitive conclusions (Baker et al., 2015). *MTT1* has already been found in distiller's and ale yeast and will likely be found to be of *S. cerevisiae* origin (Vidgren et al., 2010; Magalhães et al., 2016), while fragmentary sequences representing relatively close hits to *lager-AGT1* can be found in short reads deposited from a Tibetan and North American isolate of *S. eubayanus* (Bing et al., 2014; Hebly et al., 2015; Peris et al., 2016).

With regard to flavour production, *S. eubayanus* is characterized by a relatively modest production of acetate and ethyl esters and higher concentrations of fusel alcohols (Mertens et al., 2015), the latter of which are often described as having alcoholic and solvent-like aromas and, when present in high concentrations, are generally considered unpleasant in beer (Harrison, 1970; Meilgaard, 1982). Additionally, sensorial analysis of beers from *S. eubayanus* fermentations shows that they are characterized by the presence of strong sulphur-like flavours (Mertens et al., 2015) which normally reduce with maturation (lagering). Maybe the most characteristic flavour associated to *S. eubayanus* beers is the clove-like and/or smoky flavour derived mostly from 4-vinyl-guaiacol (4VG) (Mertens et al., 2015), occurring as a result of the decarboxylation of wort ferulic acid (Vanbeneden et al., 2008). Unlike most brewing strains, *S. eubayanus* has retained functional forms of the *FDC1* and *PAD1* genes responsible for this conversion (Baker et al., 2015; Gonçalves et al., 2016; Gallone et al., 2016).

There are no studies yet on the mechanisms behind cold tolerance in *S. eubayanus*, however, this feature is likely to be governed by similar mechanisms as in other cold tolerant *Saccharomyces* species. In *S. uvarum*, groups of genes associated with cell wall mannoproteins, ribosomal stalk, translation elongation factors, and glycolysis underwent "accelerated" evolution relative to *S. cerevisiae* (Gonçalves *et al*., 2011). In *S. kudriavzevii*, genes associated with glycerol and acetaldehyde metabolism were found to be involved in cold tolerance of this species, as well as a more efficient protein translation (Paget *et al*., 2014; García-Ríos *et al*., 2016). These findings also hold to some extent for cold-adapted *S. cerevisiae* strains (Salvadó *et al*., 2016). Although the mechanisms for yeast tolerance to cold are still poorly understood, cold

fermentation is the main feature that determines the sensorial properties of lager beer (Gibson and Liti, 2015) and thus warrants further investigation.

Despite the weaker performance of *S. eubayanus* in wort fermentations, it does possess many traits advantageous for lager brewing, such as low temperature growth (down to 4 °C), efficient maltose use, and production of desirable aroma compounds, which can be exploited for brewing innovation or can also be inherited when novel hybrids are created (Gibson et al., 2013; Hebly et al., 2015, Krogerus et al., 2015, 2016; Mertens et al., 2015). The first commercial product exclusively brewed with *S. eubayanus* has been recently released by Heineken in several countries. A strain collected close to San Carlos de Bariloche, Patagonia, Argentina (Libkind et al., 2011) was employed to brew a limited edition beer, the style of which has been referred as to 'wild lager'.

The brewing potential of other members of the *Saccharomyces* genus has yet to be explored. It may be that these species have limited ability to tolerate the stresses imposed during brewery fermentation or an inability to use wort sugars. Alternatively, the absence of certain species may be due to their geographical separation from traditional brewing areas. The discovery that *S. eubayanus* has the ability to ferment brewer's wort sets an interesting precedent, and it is likely that reports on the brewing potential of all members of the genus will soon be available. It is likely that these species, most of which have not undergone domestication, will possess traits similar to *S. eubayanus*, such as POF-production and limited ability to utilize maltotriose. The *Saccharomyces* genus as a whole has received relatively little scientific attention compared to *S. cerevisiae*. This deficiency is likely to be redressed in the future with the growing realization of its significance as a model genus (Hittinger, 2013).

Non-*Saccharomyces* yeasts in brewing

Unlike the wine industry, which values product variability arising from vintage, terroir, and other factors, the brewing industry has traditionally placed a greater emphasis on consistency and stability. This is deemed essential for brand image and customer loyalty. Critical in this regard is brewery hygiene and the use of specific, pure starter cultures for fermentation. With few exceptions, non-*Saccharomyces* yeasts in beer fermentations have been seen as detrimental to the brewing process due to associated problems related to beer turbidity, filterability, viscosity, phenolic off flavour, sourness, and other flavour profile changes (Campbell, 1996). However, attitudes in the industry may be changing, principally due to changing consumer tastes (Kellershohn & Russell, 2015). Increasing demand for traditional beer styles, alternative flavours, and low-alcohol beers has stimulated research into the potential benefits of alternative yeasts (Saerens & Swiegers, 2014b).

Volatile aroma compounds, in particular higher alcohols and esters, have a direct influence on beer quality. These compounds produced by yeasts during fermentation impart characteristic fruit and floral flavours to beer, and different aroma profiles can define particular beer styles and brands. The aromatic complexity of alcoholic beverages produced by spontaneous fermentation has been attributed to the presence of various yeast species, all of which may contribute to the final flavour profile. The functional potential of species belonging to the *Candida*, *Hanseniaspora*, *Issatchenkia*, *Kazachstania*, *Lachancea, Pichia*, *Schizosaccharomyces*, *Torulaspora*, *Wickerhamomyces*, *Williopsis*, and *Zygosaccharomyces* genera, as well as *Saccharomyces* species other than *S. cerevisiae*, has been demonstrated for wine (Jolly et al., 2014; Pérez-Torrado et al., 2017; Varela & Borneman, 2017). Individual species often produce high concentrations of particular flavour compounds, enabling them to add specific flavours to the product. *Kluyveromyces marxianus*, for example, produces relatively high levels of the rose-like flavours phenylethanol and 2-phenylethyl acetate (Carlquist et al., 2015). Other species, such as *Hanseniaspora* spp. and *Brettanomyces* spp., which are often found as contaminants in the brewing system, also produce high concentrations of these compounds (Fabre et al., 1995; Garavaglia et al., 2007; Moreira et al., 2005; Viana et al., 2009). Controlled use of these yeasts during wort fermentation may be a viable option when specific target flavours are required. *Torulaspora delbrueckii*, another common contaminant in the brewing environment has, for example, been shown to be capable of producing high levels of fruity amyl alcohol flavours (Michel et al., 2016) and is often associated with wheat beer, a style typically associated with pronounced fruit notes. *T. delbrueckii* has the added advantage of being resistant to the various stresses encountered during brewing, though individual strains vary in their ability to ferment wort sugars – a trait that may explain the variable performance observed in different studies (Canonico et al., 2016; Michel et al., 2016; Tataridis et al., 2013).

Many other non-*Saccharomyces* yeasts are unable to utilize all fermentable sugars in wort, and several studies have suggested the use of these yeasts as bioflavouring agents in co-culture fermentations with standard brewing yeast strains (Canonico et al., 2016; Saerens & Swiegers, 2014a), with the objective of producing a full-strength beer with enhanced flavour. The validity of this approach has previously been demonstrated in the wine industry (Ciani et al., 2010; Dashko et al., 2015; Viana et al., 2011; Ye et al., 2014), including for *T. delbrueckii*, commercial preparations of which are available for this purpose. In the brewing industry, reluctance to utilize non-conventional yeasts is at least partly related to the limited control the brewer has over the organisms' fermentation performances. However, adding a yeast with only a limited ability to ferment the available sugars naturally ensures that said yeast does not dominate the process. Maintaining a complex community would be particularly important and challenging in the brewing industry where the same yeast batch is often repitched in subsequent fermentations.

Yeasts with limited abilities to utilize wort sugars but that produce typical concentrations of aroma compounds are particularly desirable for the production of low-alcohol and non-alcoholic beers. Indeed, the non-*Saccharomyces* species *Saccharomycodes ludwigii* has been used commercially for this purpose for many years (Haehn & Glaubitz, 1933; Huige et al., 1990). Other species considered for this purpose include *Scheffersomyces shehatae* (formerly *Candida shehatae*) (Li et al., 2011), *Wickerhamomyces anomalus* (formerly *Pichia anomala*) (Walker 2011); *Pichia kluyveri* (Saerens & Swiegers, 2014b), and *Zygosaccharomyces rouxii* (De Francesco et al., 2015). Typically, production of alcohol-free beers involves either physical removal of alcohol from the beer or an arrested fermentation with the normal production yeast. In both cases, aroma compounds are low or absent due to their evaporation with the alcohol fraction in the former case and their lack of formation in the latter case (Brányik et al., 2012). The use of alternative, maltose-negative yeasts is therefore a useful way to produce low-alcohol beers that still retain some of the aromatic complexity of standard beers. Such yeasts will also reduce wort aldehydes, thereby removing the 'worty' taste that is often found in low-alcohol beers produced by arrested fermentation (Saison et al., 2010).

Increased demand for low-alcohol beers is one aspect of a general customer demand for more diversity in the beer market. This includes interest in beer styles that deviate from the standard flavour profile of mainstream lager beer. One such trend is a taste for sour beers. Acidic beer styles include the lambic group of beers from Belgium and the related coolship ales of North America, as well as the Berliner Weisse style found in northern Germany. Acidity in these beers is primarily due to lactic acid production by lactic acid bacteria (LAB), as well as in some cases acetic acid production by *Brettanomyces*/*Dekkera* spp. The complication of using mixed cultures in the brewery, especially where potential contaminants, such as LAB, can be avoided by using fermentative yeast species that naturally produce acids. One such species is *Lachancea thermotolerans*, a yeast that is used commercially in the wine industry to add acidity and freshness to the product. A recent report has suggested that this yeast is suitable for production of sour beers without the necessity of LAB inoculation (Domizio et al., 2016). Tested strains did not have the ability to utilize maltotriose, the second most abundant sugar in wort, but they were otherwise found to be suitable for single-culture beer fermentation. In particular, *L. thermotolerans* did not produce off flavours that would be expected with *Brettanomyces*/*Dekkera* spp., for example. *Hanseniaspora uvarum* is another yeast with acidifying power (in this case through the production of acetic acid) (Cabranes et al., 1996). As the species is a common contaminant in brewery fermentations, it may be assumed that it is tolerant of the typical stresses encountered in the system and may also have potential for sour beer production.

Arguably the most successful non-*Saccharomyces* yeasts involved in beer fermentation are the *Brettanomyces*/*Dekkera* species. These yeasts, particularly *Brettanomyces bruxellensis* and *B. anomala*,

are essential in the production of lambic-style beers, where they contribute flavours that are not normally produced by *Saccharomyces*. In particular, volatile phenolic compounds and organic acids produced by *Brettanomyces* spp. can impart smoky, barnyard, spicy, and medicinal flavours, collectively described as 'Brett' character and with the pleasantness determined by the concentration and consumer tastes (Steensels et al., 2015). Interestingly, the *Brettanomyces* spp. also have the ability to reveal masked flavours through their production of β-glucosidases. The primary function of these enzymes is hydrolysis of cellobiose, a feature that may explain the ability of these yeasts to survive for extended periods in the oak barrels used for lambic beer fermentation. These enzymes also have the effect of liberating glycosidically-bound flavour compounds, thus adding complexity to the flavour profile of wines and beers (Daenen et al., 2008). Such changes have been seen in wines and lambic beers, but as they can act on hop glycosides, they are potentially relevant to the majority of beer styles. Such reactions can enhance the levels of linalool (imparting citrus, floral, and aniseed flavours) and methyl salicylate (imparting wintergreen, mint, and spice flavours) (Winterhalter & Skouroumounis, 1997). A number of other species are known to have this activity, including some *Saccharomyces* yeast (Sharp et al., 2017) and several non-*Saccharomyces* yeast including *Debaryomyces* spp., *Hanseniaspora* spp., and *Pichia terricola* (formerly *Issatchenkia terricola*) (Steensels & Verstrepen, 2014). Thus far, these reactions have mainly been studied in relation to their impact on wine, and it remains to be seen if the hop glycoside content of beer is high enough for this activity to have a significant impact on flavour (Sharp et al., 2017).

The use of non-*Saccharomyces* yeasts is a natural way to introduce diversity to beers on the market. The mainstream brewing industry has, however, been slow to take advantage of the increased functionality offered by alternative yeasts. These organisms have been more enthusiastically embraced in the wine industry where non-*Saccharomyces* yeasts are a normal part of the microflora during fermentation. Modern breweries maintain high levels of hygiene, and brewers are understandably reluctant to introduce foreign strains with the potential to cause contamination. Another issue is how these new yeasts can be handled in a controlled manner to achieve desired beer characteristics. However, in certain cases, the use of alternative yeast strains may be the simpler option: for example, using *L. thermotolerans* to produce sour beer may be simpler than maintaining a co-fermentation with yeasts and bacteria.

The successful application of non-conventional yeasts in brewing may, in some cases, require important changes to process conditions. One such condition is oxygen availability. In standard brewing, wort is aerated before or at the time of pitching to support initial growth of the yeast population. Thereafter, fermentation proceeds without additional oxygen. This may not be an option when certain non-conventional yeasts are employed. *Torulaspora delbrueckii*, for example, is dependent on a low level of oxygen to support fermentation (Alves-Araújo *et al*., 2007), and *Brettanomyces* spp., despite being able to

ferment anaerobically, are more efficient when low levels of oxygen are introduced (Aguilar-Uscanga *et al*., 2003). In the case of co-cultivation of *Saccharomyces* brewing yeasts and non-conventional yeasts for the purpose of bioflavouring, this dependence on oxygen may have a positive role in controlling the growth of the latter, thereby ensuring successful completion of fermentation by the former.

A further complication is that the list of yeasts that are unequivocally and generally recognized as safe (GRAS/QPS) for use in food production is a short one (Ricci et al., 2017), and further testing may be necessary to allay fears regarding consumer safety.

Generation of new ale and lager yeast through hybridisation

In addition to the traditional yeast hybrids that have been used extensively by the brewing industry since their isolation by Hansen and Elion in the 1880s, there is an increasing interest in new brewing yeast hybrids generated by *de novo hybridisation*. The breeding of brewing yeasts has been carried out for decades in attempts to generate unique strains and improve fermentation performance (Johnston, 1965, Russell *et al.*, 1983, Spencer & Spencer, 1977). However, because most industrial brewing yeasts have been domesticated (Gallone et al., 2016, Gonçalves *et al*., 2016), many have, characteristically lost the ability to sporulate and sexually reproduce (Bilinski *et al*., 1986, Gallone *et al*., 2016). This restricts the use of certain classical breeding techniques, where haploid cells of opposite mating type derived from spores are brought together and allowed to fuse. Nevertheless, the spore-to-spore mating approach has been successfully applied to a wide range of strains and species. Garcia-Sanchez and co-workers (2012) describe how crossing rare viable spores of a *S. pastorianus* strain with those of a *S. cerevisiae* ale strain yielded hybrids with improved growth at higher temperatures and greater tolerance to higher ethanol concentrations. The availability of *S. eubayanus* from 2011 onwards permitted the recreation of the *S. cerevisiae* x *S. eubayanus* interspecies hybrid, which until then had only existed in the form of the traditional lager yeast strains used for centuries in the brewing industry. Hebly et al., (2015) showed how the psychrotolerant phenotype could be inherited by hybrids of the *S. eubayanus* type strain and a laboratory strain of *S. cerevisiae* after spore-to-spore mating. In the same year Mertens and co-workers (2015), also by mating spores, produced a set of 31 hybrids by crossing spores of six different ale strains with *S. eubayanus*. Many of these hybrids possessed a broader temperature tolerance and produced a more diverse aroma compound profile than their parent strains. In a preceding study, Steensels and co-workers (2014) had used a variant of spore-to-spore mating of three genetically diverse *S. cerevisiae* strains to generate 46 hybrids, many of which produced increased levels of 3-methylbutyl acetate (banana/pear aroma) compared to the parent strains. Here, the parent strains were first screened for heterothallism, and spore clones exhibiting stable mating types were used for hybridisation.

Various strategies have been developed to overcome the limitation of low fertility in traditional brewing yeasts, the most extensively used of which are rare mating and protoplast fusion. During rare mating, one exploits the fact that spontaneous loss of heterozygosity at the mating type locus can occur at low frequencies (10^{-4}), resulting in the formation of diploid (or potentially higher ploidy) cells with a single mating type (Hiraoka *et al*., 2000). This procedure can also enable mating among yeast strains that are unable to sporulate. This approach has been used by Choi and co-workers (2002) to generate a dextrin-fermenting brewing yeast by rare mating a strain of *S. cerevisiae* ("var. *diastaticus*") with a *S. cerevisiae* ale strain, while Sato and co-workers (2002) used rare mating to cross a "*S. bayanus*" strain with a *S. cerevisiae* ale strain to yield a more cold-tolerant hybrid. More recently, Krogerus and co-workers (2015, 2016) used rare mating to generate hybrids between a *S. cerevisiae* ale strain and *S. eubayanus* with improved fermentation performance and higher aroma formation. While rare mating allows for the hybridisation of strains with low fertility, the hybridisation frequencies are typically low, and the parent strains require selection markers (e.g., auxotrophies) so that hybrids can be isolated from the population of parent cells. In an attempt to increase the hybridisation frequency of rare matings, Alexander and co-workers (2016) described a Hybrid Production (HyPr) technique that can be used to force mating-type change in diploid cells by transformation with a plasmid carrying the *HO* gene under the control of an inducible promoter, hence bypassing the need for spontaneous loss of heterozygosity prior to hybridisation. Techniques that leave no trace of foreign DNA in the genome, such as HyPr and CRISPR/Cas9 genome editing, seem to have been given a boost by recent decisions by the United States Department of Agriculture to not regulate such organisms (Ledford, 2016; Waltz, 2016; Lee, 2017), but it is likely that they would still be viewed sceptically by industry, consumers, and other jurisdictions.

De novo yeast hybrids have been used to successfully improve beer fermentation in a number of respects, including faster fermentation rates, increased aroma formation, and higher stress tolerance. These results have been seen in both experimental-scale (150mL - 2L) and pilot-scale (50L) fermentations using wort strengths of 12-15°P (Krogerus *et al*., 2015; Mertens *et al*., 2015). The improvement in fermentation performance observed in interspecies yeast hybrids relative to their parents can be justified based on improvements on sugar utilisation rate and temperature tolerance. Although it is likely that maltose transporters were inherited from both parent strains in lager yeast hybrids, the origin of the transporters able to carry maltotriose is still a matter for debate (Baker et al., 2015). In *de novo* hybrids, it is likely that maltotriose utilisation is a property transferred by the *S. cerevisiae* parent, considering that none of the *S. eubayanus* strains characterized so far have the ability to use this sugar (Krogerus *et al*., 2015, 2016; Hebly *et al*., 2015; Mertens *et al*., 2015). However, newly-found *S. eubayanus* strains (Bing *et al*., 2014; Peris *et al*., 2014; Gayevskiy and Goddard 2016) remain to be tested for maltotriose utilisation. The main contribution of *S. eubayanus* for the fermentation performance of artificial hybrids seems to be

cold tolerance (Krogerus *et al*., 2015, 2016; Hebly *et al*., 2015; Mertens *et al*., 2015). The combination of superior sugar transport with cold tolerance likely enabled the hybrids to outperform the parents at the low temperatures used for lager brewing (8-15 °C; Krogerus et al., 2015, 2016; Mertens et al., 2015). *De novo* interspecific hybrids have even displayed similar fermentation efficiencies to *S. pastorianus* strains currently used for commercial beer production (Krogerus et al., 2015; Mertens et al., 2015).

Krogerus et al., (2016) further revealed that the ploidy level influences fermentation performance, as hybrid strains with higher DNA content were superior to lower ploidy hybrids in the fermentation of wort at 15 °C. These 1.5L fermentations were conducted in both standard (15°P) and very high gravity (25°P) wort and the relative improvement in fermentation performance was seen throughout the fermentations, with the exception of the first 24 hours when fermentation is driven largely by monosaccharide utilization. Furthermore, a link between the fermentation performance of hybrids and their sugar consumption abilities was observed, as strains fermenting fastest also consumed maltose and maltotriose fastest. Since the uptake of maltose and maltotriose tends to limit fermentation capacity during brewing (Alves et al., 2007; Rautio & Londesborough 2003), higher ploidy hybrids would result in a greater number of maltose/maltotriose transporter genes in hybrid genomes, which could account for improved uptake of these sugars. Similarly, the allotetraploid lager yeast strains from group II tend to perform better that the allotriploid ones from group I, although exceptions can be found (Gibson *et al*., 2013; Magalhães *et al*., 2016).

In addition to attempting to increase fermentation performance, many studies on yeast hybrids have focused on attempting to increase the formation and diversity of aroma-active compounds (Bellon *et al*., 2011, 2013, Krogerus *et al*., 2015, 2016, Mertens *et al*., 2015, Mukai *et al*., 2001, Steensels *et al*., 2014). Studies on intraspecific *S. cerevisiae* hybrids have demonstrated the possibility of increasing the formation of both ethyl and acetate esters in comparison to the parent strains (Mukai *et al.*, 2001, Steensels *et al.*, 2014). Steensels and co-workers (2014) revealed that an increase in 3-methylbutyl acetate formation of up to 45% could be obtained by hybridisation, and that heterosis was particularly prevalent in outbred hybrids. The aroma spectrum of natural lager yeast hybrids is rather limited (Gibson et al., 2013; Mertens et al., 2015). However, interspecies hybridisation has shown to have potential for increasing the aromatic diversity in 50L pilot-scale fermentations (Mertens *et al*., 2015). In artificial hybrids, the aroma profiles ranged from worst- to best-parent levels, with several of the hybrids producing higher concentrations of aroma compounds than either of their parents (Krogerus *et al*., 2015; Mertens *et al*., 2015). Similarly to temperature tolerance, aroma profile can be controlled based on the relative contribution of parental DNA (Krogerus *et al*., 2016). Tetraploid hybrids produced higher concentrations of ethyl and acetate esters than the triploid and diploid hybrids, likely due to increased copy number and transcription of several key genes related to the synthesis of ethyl and acetate esters (Krogerus *et al*.,

2016). Some compounds produced by yeasts are not necessarily desirable, and hybridisation strategies may accentuate their synthesis. Increased production of compounds like ethyl acetate, which is unpleasant at high concentrations (Steensels *et al*., 2014), and vicinal diketones (Krogerus *et al*., 2016) by hybrid strains have been reported. However, hybridisation or hybridisation followed by sporulation and isolation of spore clones has been used in efforts to remove unpleasant flavours. Such approaches proved efficient for the removal of 4-vinylguaiacol (Gallone *et al*., 2016, Krogerus *et al.*, 2017, Tubb *et al*., 1981), H_2S (Bizaj *et al*., 2012) and ethanethiol (Magalhães *et al*., 2017). While much remains to be learned, the knowledge obtained so far can be applied for the design of new hybrid strains by careful selection of parents. Before they may be utilized at industrial scale, further characterization of *de novo* hybrid yeast performance at pilot scale will be necessary. To date, only one such study has been carried out (Mertens *et al*., 2015). Further sensory analysis of the resultant beers will also be necessary to identify any flavour attributes, either positive or negative, that might differentiate the beers from standard lager beers.

Research on *de novo* hybrids for brewing purposes has been triggered by the discovery of *S. eubayanus*. As research advances, it has become evident that the main contribution of the currently available *S. eubayanus* strains to the hybrid phenotypes is the cold tolerance. Due to the limited genetic diversity and the restricted geographical ranges of available *S. eubayanus* strains, one may consider the use of other cold tolerant *Saccharomyces* species in hybridisation experiments for brewing purposes. The feasibility of this approach is supported by the fact that a second *Saccharomyces* species, which also expresses a cold-tolerant phenotype, is associated with beer fermentation but, like *S. eubayanus*, is only found as a hybrid in partnership with *S. cerevisiae*. *Saccharomyces kudriavzevii*, a yeast species frequently associated with oak forests mainly in Europe and Eurasia, has little history of domestication in association with the beer fermentation process. This is probably due to the relatively low ethanol tolerance of *S. kudriavzevii* in comparison with other *Saccharomyces* species. *S. kudriavzevii* shows weak or no growth above 5% ethanol (Belloch et al., 2008). Competitive exclusion of *S. kudriavzevii* by other mesophilic and/or more ethanol-tolerant *Saccharomyces* species has been experimentally demonstrated in laboratory mixed cultures (Sampaio and Gonçalves 2008; Arroyo-López et al., 2011). However, as already mentioned for *S. eubayanus*, *S. kudriavzevii* contributes to some hybrid brewing strains, rather than as a pure lineage. Hybrid strains combining the genomes of *S. cerevisiae* and *S. kudriavzevii* have been isolated and characterized from fermenting environments related to beer and seem to be common in Belgian-style beers (González et al., 2008). With the implementation of genome sequencing studies, many strains originally assumed to be *S. cerevisiae* are now being recognized as *S. cerevisiae x S. kudriavzevii* hybrids. At least one quarter of 24 brewing strains regarded as *S. cerevisiae* were found to be in fact *S. cerevisiae x S. kudriavzevii* hybrids (González et al., 2008). Half of these hybrids were recovered from Belgian speciality beers from Trappist monasteries (Trappist beers). Bottle re-fermentation or conditioning is a common practice in the

production of these types of beers (van Landschoot et al., 2005), which allows adjusting and/or modifying the final flavour of beer, also known as bioflavouring (Vanderhaegen et al., 2003). These results suggest that a large fraction of brewing strains may correspond to *S. cerevisiae x S. kudriavzevii* hybrids.

The potential of *de novo S. cerevisiae* interspecific hybrids with *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, and *S. uvarum* has been demonstrated in winemaking conditions (Bellon *et al*., 2011, Bellon *et al*., 2013, Bellon *et al*., 2015, Lopandic *et al*., 2016) and also recently for bioethanol production (Peris et al., 2017b). Species like *S. kudriavzevii* and *S. uvarum* have been shown to possess tolerance towards low fermentation temperatures (Gonçalves *et al*., 2011, López-Malo *et al*., 2013), and they could feasibly act as alternatives to *S. eubayanus*. Indeed, which lineages of *Saccharomyces* were historically tapped by European brewers for domestication may be an accident of biogeography, rather than a lack of brewing potential: considerable lineage- and population-level diversity remains unexplored in each of these species (Hittinger et al., 2010; Almeida et al., 2014; Leducq et al., 2016; Peris et al., 2016; Flores et al., 2017). Future research into these new species and lineages may unlock traits and flavours inaccessible in current industrial brewing strains.

The hybrid genome

Hybrid sterility and fertility

Hybrids can clearly exhibit improvements of desired traits over the parents, as described above, but the issue of hybrid sterility impedes our understanding of the genetics of such improvements, as well as the genetics of interactions between the genomes. As mentioned above, there have been several approaches towards dealing with existing hybrids using rare viable spores (Gjermansen & Sigsgaard 1981), or creating new hybrids with various mating schemes using spore to spore mating (Naumov 1987; Steensels et al., 2014), complementation of auxotrophies (Naumov et al., 1995a; 1995b), or stable heterothallic derivatives (Greig et al., 2002). Diversity could be generated in the parental strains prior to hybridization and effects on phenotypes in the hybrid inferred but this is like selecting desired traits in mules by phenotype choice in the horse and donkey parents. Any genetic interactions in the hybrid may not be predictable or determinable. Nevertheless, this has been a successful approach in creating new brewing hybrids (Steensels et al., 2014).

Hybrid sterility in *Saccharomyces* is the basis of the biological species definition (Naumov 1987; Greig, 2009; Louis, 2011). There are translocations between species and populations that have been demonstrated to be involved in sterility in some cases. Although no dominant B-D-M (Bateson – Dobszhansy – Muller) incompatibilities have been found between species (Greig et al., 2002) and there is

little evidence of recessive nuclear incompatibilities (Greig 2007), there are known incompatibilities between nuclear gene variants and mitochondrial variants between species (Lee et al., 2008; Chou et al., 2010). Condition-specific B-D-M incompatibilities have been found between different *S. cerevisiae* strains that affect fitness (Hou et al., 2015), and therefore, there are likely to be some between species. The final cause of sterility is simply sequence divergence preventing proper meiotic recombination and chromosome segregation (Hunter et al., 1996; Chambers et al., 1996; Greig et al., 2003; Liti et al., 2006; Louis 2011; Hittinger 2013). The sterility due to sequence divergence can be overcome by providing homologous chromosome partners in meiosis, which can be accomplished by increasing ploidy. Tetraploid hybrids are fertile and exhibit high spore viability (Greig et al., 2002). If variation is incorporated into the two parental diploid species, then the resulting diploid hybrids from the tetraploid spores will each have a unique combination of recombinant parental species genomes. This approach could then allow genetic mapping to be performed, even on complex traits. With the appropriate manipulations already in use to create the hybrids and tetraploids, further crosses and backcrosses can be made, opening up sterile hybrids to classical genetic analysis. The future of new hybrid strain development will likely use breeding genetics in this way.

Interaction between subgenomes of interspecies hybrids

It is possible that many of the traits of hybrids are simply combinations of independent traits of each parent, such as cryotolerance coming from *S. eubayanus* and maltotriose utilisation from *S. cerevisiae*. However, some traits are better than the combination of the two parents, and clearly there must be interactions occurring between the genomes. One of the most severe is the nuclear – mitochondrial incompatibilities that are involved in reproductive isolation, as described above. In a study of protein complexes in newly generated *S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. mikatae* (Piatkowska et al., 2013), chimeric complexes were found in several cases that exhibited different phenotypes in different conditions. They also demonstrated an advantage of the chimeric complex of the hybrid in at least one case. With regards to aroma formation, studies of both traditional and *de novo*-generated lager yeast hybrids have indicated that the dosage and expression levels of genes involved in the synthesis of these compounds seem to correlate with the amounts produced (Krogerus *et al*., 2016, Van den Broek *et al*., 2015). This is a phenomenon that can be taken advantage of when choosing a particular hybridization approach for a particular hybrid phenotype (Krogerus *et al*., 2016). However, little is still known about transregulation and subgenome cooperation in brewing yeast hybrids. With classical and quantitative genetic analysis available for those hybrids that can go through a tetraploid intermediate, the interactions between the subgenomes will be amenable to dissection. As more knowledge on the link between genotype and phenotype of hybrids becomes available, it will allow for targeted selection of parental strains for breeding and hybrid screening.

Mitochondrial inheritance

The powerhouse of the cell contains its own genome that encodes a handful of genes in *Saccharomyces* that have not been transferred to the nuclear genome. Mitochondrial genome sizes range from below 65 kbp in *S. eubayanus* to above 85 kbp in *S. cerevisiae* (Foury et al., 1998; Baker et al., 2015). They have low GC-content (below 20%), have low protein-coding potential, and are littered with selfish elements and introns. Even so, the fact that all mapped incompatibilities preventing the meiotic fertility of *Saccharomyces* interspecies hybrids involve at least one mitochondrial gene implies that mitochondrial genomes are functionally important (Lee et al., 2008; Chou et al., 2010). The inheritance of the mitochondrial genome in industrial and synthetic hybrids also appears non-random. In synthetic hybrids, the mitochondrial genome rapidly stabilizes to a single, sometimes recombinant, haplotype (Marinoni et al., 1999; Peris et al., 2017b). Environmental conditions can dramatically influence which parent's mitochondrial genome is retained, further suggesting an important functional role (Hsu & Chou, 2017). Lager yeasts inherited *S. eubayanus* mitochondrial genomes that contain a snippet of introgression from *S. uvarum* at a known recombination hotspot in *COX2* (Peris et al., 2014). Nearly all known *S. cerevisiae x S. kudriavzevii* hybrids have retained the mitochondrial genome of *S. kudriavzevii* (or a recombinant derivative), even though they tend to have lost several *S. kudriavzevii* nuclear chromosomes (Peris et al., 2017a). Thus, retention of the mitochondrial genomes from the non-*S. cerevisiae* parent may confer a selective advantage or desirable properties in industrial fermentation conditions.

Hybrid genome stability

De novo hybrids tend to display genetic instability post-hybridisation (Kumaran *et al*., 2013, Pérez-Través *et al*., 2012, Selmecki *et al*., 2015; Peris et al., 2017b)., and this could pose a problem for the brewing industry, where yeast is often reused multiple times and consistency is required. However, traditional lager yeast genomes have also been shown to contain chromosome losses and intrachromosomal translocations, sequence divergence, and chromosome copy number variations (van den Broek et al., 2015). This instability of newly-formed brewing hybrids could also be taken advantage of in adaptive evolution experiments (Dunn *et al*., 2013, Piotrowski *et al*., 2012; Peris et al., 2017b), as the natural lager yeast genome has been shown amenable to change via evolutionary engineering (Blieck *et al*., 2007, Ekberg *et al*., 2013). By subjecting hybrids to different environmental conditions, it could be possible to target and improve on specific phenotypes.

Conclusion

Recent years have seen a greater customer demand for diversity in commercially available beers. The beer-drinking public now has a greater interest in the brewing process and in the many styles of beers that are available or have been available in the past. There is also a growing appreciation of the role that yeasts play in determining these styles. This interest in brewing yeasts has coincided with the greater availability of techniques for their study. Genome analysis in particular is improving our understanding of how ale and lager-brewing yeasts have evolved to exploit their respective fermentation environments, while promising to simultaneously satisfy the brewer's demands for quality beer. Our improved understanding of brewing yeast biology allows for better selection of strains for particular processes and the selection of appropriate traits for development. The emergence of liquid and colony handling robots further allows the possibility of performing high-throughput phenotyping assays on hundreds of strains simultaneously, as done in a number of recent large-scale breeding-related studies (Gallone *et al.*, 2016, Mertens *et al.*, 2015, Snoek *et al.*, 2015, Steensels *et al.*, 2014).

The discovery of *S. eubayanus* has clarified the development of the interspecies hybrid *S. pastorianus* (though much remains to be discovered about this unique organism), and it has inspired a number of successful attempts to recreate the *S. cerevisiae* x *S. eubayanus* hybridization event. These efforts are expected to increase the genetic diversity of strains available for lager brewing, thereby creating further diversity in the beer market. Many hybridisation approaches, whether for ale or lager yeasts, can satisfy the market demand for diversity, while respecting customers' scepticism of GM technology applied to brewing as well as local legislation regarding the use of GMOs.

Brewing yeast research has, in the past, made a direct contribution to our fundamental understanding of biology (Barnett & Lichtenthaler, 2001). It may be expected that the recent resurgence of interest in brewing yeasts could similarly contribute to our general understanding of the natural world. There is, for example, a growing realisation that many species, including our own (Simontti et al., 2016), have been influenced by hybridisation, and *S. pastorianus* could serve as a model organism for the study of hybrid genome function. Also, the search for alternative brewing yeasts (*S. eubayanus*, *L. thermotolerans*, and others) in nature will greatly improve our understanding of the biogeography and ecology of yeast species and may inspire a greater appreciation of the potential importance of yeast diversity for the biotechnological processes of the future.

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CTH declares the following conflicts of interest: the Wisconsin Alumni Research Foundation (WARF), William G. Alexander, David Peris, and CTH have filed a provisional patent application on HyPr; commercial use of certain strains is subject to licencing agreements with WARF; all technologies and strains are freely available for non-commercial academic research.

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