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Review

Designing New Yeasts for Craft Brewing: When Natural Biodiversity Meets Biotechnology

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Abstract: Beer is a fermented beverage with a history as old as human civilization and its productive process has been spread all around the world becoming unique in every country and iconic of entire populations. Ales and lagers are by far the most common beers; however, the combination of raw materials, manufacture techniques and aroma profiles are almost infinite, so it is not surprising to notice that there is a large amount of different beer styles, each of them with unique characteristics. Nowadays, diversification is becoming increasingly important in the brewing market and the brewers are continuously interested in improving and extending the already wide range of products, especially in craft brewery. One of the major components that can have a deep impact on the final product is yeast, since it is able to convert carbohydrates in wort, especially maltose and maltotriose, into ethanol, carbon dioxide and other minor aroma-active compounds. *Saccharomyces cerevisiae* (top-fermenting yeasts used to produce ales) and *Saccharomyces pastorianus* (cryotolerant bottom-fermenting hybrids between *S. cerevisiae* and *Saccharomyces eubayanus* responsible for the fermentation of lagers) are most used in breweries. However, an increasing number of different yeast starter cultures are commercially available, to improve the production efficiency also at relative low temperatures and to obtain desirable and diversified aroma profiles avoiding undesired compounds. Four main genetic engineering-free trends are becoming popular in craft brewing yeast development: 1) the research for novel reservoirs as source of new performant *S. cerevisiae* yeasts; 2) the creation of synthetic hybrids between *S. cerevisiae* and *Saccharomyces* non-*cerevisiae* in order to mimic lager yeasts by expanding their genetic background; 3) the exploitation of evolutionary engineering approaches; 4) the usage of non-*Saccharomyces* yeasts either in co-coculture or in sequential fermentation with *S. cerevisiae*. In the present work we summarized pro and contra of these approaches and provided an overview on the most recent advances on how brewing yeast genome evolved and domestication took place. Finally, we delineated how the correlations maps between genotypes and relevant brewing phenotypes can assist and further improve the search for novel craft beer starter yeasts.

Keywords: craft brewing; *Saccharomyces cerevisiae*; *Saccharomyces eubayanus*; hybrids; 4-vinyl guaiacol; non-conventional yeasts; evolutionary engineering; artisanal fermented food; natural biodiversity.

1. Introduction

Human history is woven with brewing activity ever since the beginning of civilization in the Neolithic period [1-3]. Since then, the main ingredients are not significantly changed: water, yeast, cereals and hops. Nowadays, productive process includes basically the phases of malting, in which cereals (mainly barley) are converted in malt; mashing, that permits to obtain wort; and fermentation, that finally generates beer. Looking at the ingredients and the productive process, beer appears to be a highly consolidated and sufficiently known product. This consideration is, however, disproved thinking of all the sciences behind brewing process: microbiology, chemistry, agronomy but even

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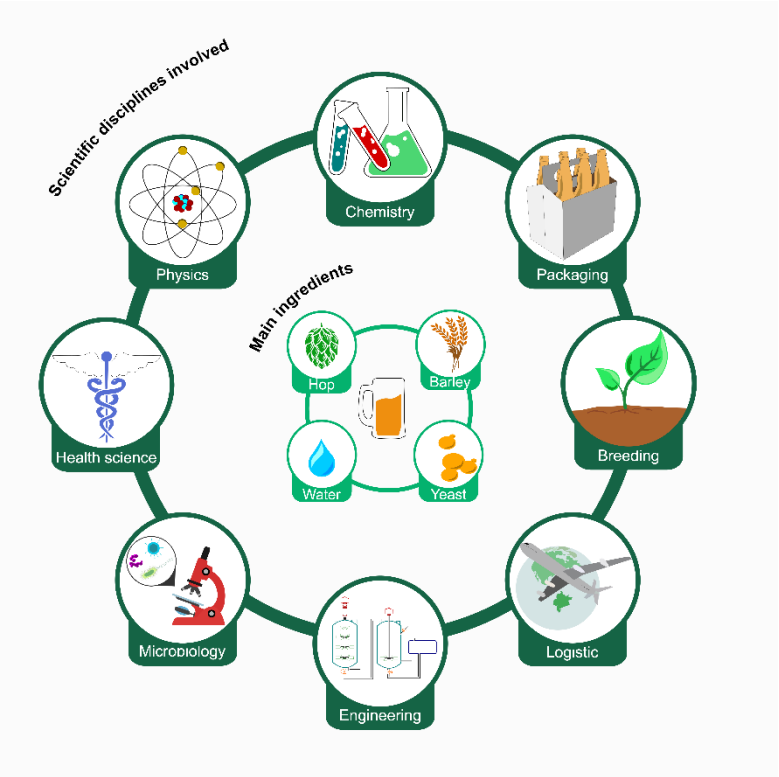
logistic, marketing, process engineering and health science cooperate to obtain high quality and versatile products competitive on the market (**Figure 1**).

Figure 1. Multi-disciplinary perspective for challenging the brewing complexity.



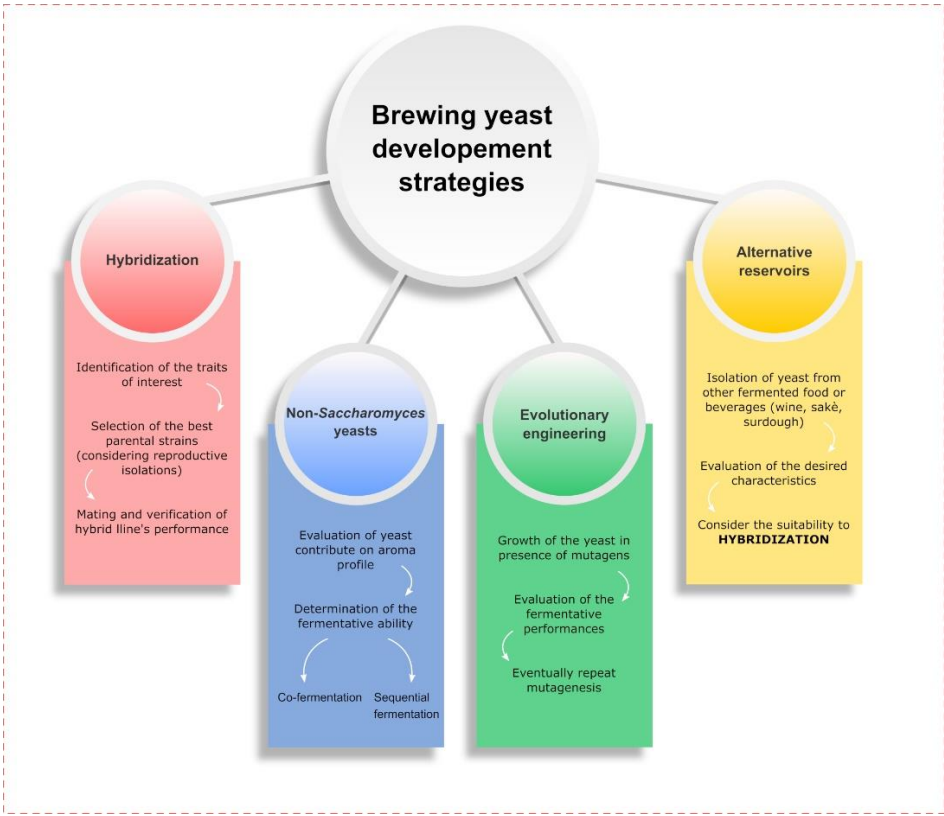
It is precisely because of the vastness of the knowledge to which we can nowadays tap into that is complex to achieve a unique and fully accepted definition of beer without demanding clarifications. Conventionally, the term "beer" refers to as a broad pattern of fermented beverages based on cereals or, in a more limited way, as the hopped drink obtained from liquefied starch after fermentation accomplished with specific strains of *Saccharomyces cerevisiae* yeasts. Ale, lager, porter, stout, lambic, waisse and many other words can be found beside the general "beer" term nowadays in every market. Each one of them refers to specific beer products with peculiar visive, sensorial and chemical-compositional properties, such as bitterness, associated with compounds of hops and expressed in International Bitterness Unit; alcohol-by-volume (ABV), indicated as percentage of volume in the product; color, measured according to Standard Reference Method (SRM) from the American Society of Brewing Chemist (ASBC) or to European Brewing Convention as an alternative outside the US; and original and final gravity (OG and FG) [4]. However, the principal separation criterion accepted nowadays for beer classification is based on the type of fermentation performed during the brewing process. Based on this parameter three macro-categories of beer are predominant: ale, lager and lambic. Ale beers are produced utilizing selected yeast strains of top-fermenting *S. cerevisiae* at fermentation temperature of 15°C-25°C. Lager style beer involves instead bottom-fermenting yeasts strains of allopolyploid yeast *Saccharomyces pastorianus* (a hybrid species between *S. cerevisiae* and *Saccharomyces eubayanus*) in a process conducted at temperature of 8°C-12°C [5]. Finally, lambic style beer is characterized by a spontaneous fermentation because, originally, it was performed without any specific starter cultures, but just exposing the wort to the air letting it colonized by wild yeasts and bacteria. The result is a beer with very particular aroma, even completely different from ale or lager. Further, other specialty beers are Trappist beers which entail a secondary *S. cerevisiae* fermentation in the bottle and wheat beers which are brewed with a large proportion of wheat. Apart from these specialties mainly diffused in Belgium and the UK, in the past decades few macro-breweries dominating the global beer market promoted strong homogenization of products towards the mild lager beer styles, which were appreciated by a wide consumers audience for their fresh

flavor profile, i.e. lack of ester-derived fruity/floral aroma, resulting mainly from fermentation at low temperatures. These lager products represent the 90% of the beer market [6].

Starting from 1980s, an increasing trend in food and beverage industry was to evolve its own product not only to appeal as many consumers as possible, but even to surprise and arouse curiosity for one's own proposal and brand [6] or to better fit specific local tastes [7]. In addition, global habits of food consumption changed towards increased demands for healthier food and drinks [8]. In agreement with these trends, the consumption of beer is decreasing in Northern America and Europe. In this highly competitive scenario, segmentation of beer market provided an avenue for business to remain viable [9]. In particular, high-income and sophisticated consumers looked for a variety of local beer products with high quality ingredients and high level of "beverage culture" [10,11]. The most dynamic and iconic sector is certainly that of craft brewing. Craft beer is gaining a rapid increasing in popularity in the mainstream markets of Northern America and Europe, claiming a larger segment in beer sector every year. In 2014 the craft beer sector earned nearly $20 billion in the US, where craft market share grew from 5.7% to 12.3% in in the time span from 2011 to 2016 [12]. The awareness about craft beer seems rather low in Europe [13], but the number of craft breweries is constantly growing in several countries, such as UK, Italy, France and Belgium [14]. Even if craft beers are hyper-differentiated products which can mix characteristics of different styles [15], they exhibit some common aspects. Generally, craft beer is produced by small, independent and traditional breweries [16] and it is usually unfiltered, unpasteurized beverage, without additional nitrogen or carbon dioxide pressure and re-fermented in bottle. However, the attitude at brewing process is associated with a flexible, adaptable and experimental approach, which includ8es the use of alternative ingredients such as tobacco, tomatoes, coffee, cacao, fruit and a range of spices [17]. One strategy in response to the growing success of craft beer is for macro-brewers to produce a craft(-style) beer themselves, making the search for novel technical innovations to produce versatile products even more compelling [11].

Fermentation plays a key role in determining flavorful alternative products, as yeast metabolism strongly affects not only alcohol production from maltose and maltotriose, but also flavor and aroma composition. Pyruvate produced by yeast glycolysis is not only the precursor of ethanol and carbon dioxide, but also provides carbon skeletons for the synthesis of amino acids, which are involved in the production of diketones and several aroma compounds such as sulfur-containing compounds, esters and higher alcohols [18]. Additionally, yeasts can modify the phenolic compounds present in wort, releasing volatile organic compounds (VOCs). Therefore, fermentation phase represents the widest space for beer innovation and diversification in the brewing process. In the era of low-cost sequencing technologies, genomics and transcriptomics data are accumulating to depict the trajectories of yeast genome evolution and to chart maps between genome landscape and industrially interesting phenotypes. This review summarizes the main knowledge on beer yeast genomics and describes how this information can drive and accelerate the selection of novel yeast starters for brewing. In this context, four main innovation trends were delineated in beer fermentation, including i) the mimics of lager yeasts by the creation of synthetic hybrids between *S. cerevisiae* and cold-tolerant *Saccharomyces* non-*cerevisiae* strains; ii) the evolutionary engineering techniques to improve fermentative performance in high brevity wort and recently also to enhance flavor; iii) the search of new performant *S. cerevisiae* yeasts from alternative bioreservoirs such as artisanal fermented food; iv) the usage of non-*S. cerevisiae* yeasts as flavoring agents (**Figure 2**).

Figure 2. Main trends in brewing innovation driven by knowledge on molecular mechanisms underpinning beer-relevant phenotypes.



2. Brewing yeasts though the lens of genomics

The role of the yeasts in alcoholic fermentation has been unknown until Louis Pasteur clarified the process in his book "Etudes sur la Bière" [19] and Emil Hansen isolated the first pure culture of brewer's yeast, "Carlsberg Yeast Number 1", on solid media. Successively, the use of pure yeast cultures in the beer production, pioneered by Christian Hansen, certainly improved the consistency and quality of beer, but on the other hand this strategy and Hansen's policy of donating the Carlsberg Brewery's yeast strains to other brewing companies limited the biodiversity of the brewing yeasts. Before the brewing industrialization, individual strains have been conserved by individual breweries and even households [20].

Brewers traditionally distinguish ale and lager brewing yeasts, according to their use for the production of ale and lager beers. Ale yeasts, classified as top-fermenters, tend to float to the top of the vat at the end of the fermentation, whereas cold-tolerant lager yeasts, bottom-fermenters, sediment to the bottom. Generally, the ale fermentation is carried out at relatively high temperature (15-26°C), while lager fermentation at lower temperature (8-15°C). Advances in next-generation sequencing and phenomics technologies have recently allowed the analysis of an increased number of ale and lager genomes and their related industrially relevant phenotypes. This comparative frameshift provided new insights on how brewing yeasts evolved and reveled the main domestication events which triggered different evolutionary trajectories and made ale and lager yeasts adapted to specific industrial niches. Below, we summarized the main recent developments in phylogenomics and adaptive evolution of ale and lager yeasts, pointing out this information can be useful in understanding the genetic signatures of brewing traits (**Figure 3**).

Figure 3. Marker assisted selection of natural variants harboring brewing relevant traits.

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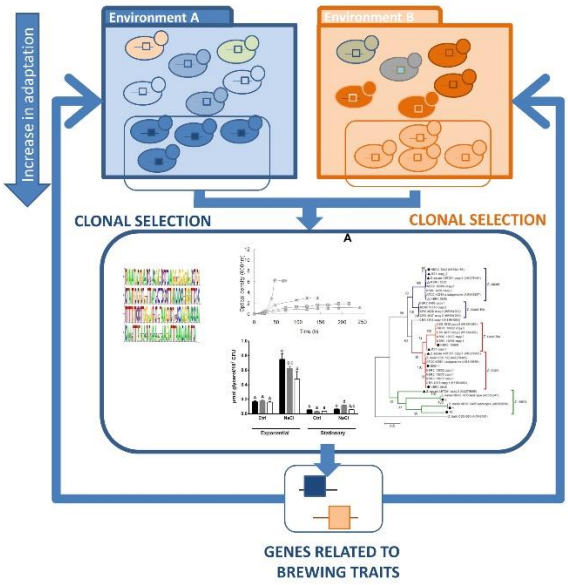

2.1. Saccharomyces cerevisiae Ale Yeasts

Comparative genomic studies demonstrated that most of the ale beer strains were genetically distinct from wild stocks, and mainly clustered into two independent lineages, called Beer 1 (which consists of three separate Belgium/Germany, Britain, and the United States strains), and Beer 2 (which contains yeasts originating from Belgium, the United Kingdom, the United States, Germany, and Eastern Europe) [21,22]. Generally, *S. cerevisiae* ale strains exhibit large-scale variations in genome structure, including changes in ploidy and large segmental duplications or copy number variations, due to the adaptation to the specific brewing niche. Most of the small structural genome variations are commonly located in telomeric and sub-telomeric regions, that represent typically hotspots for evolution. Unlike wild *S. cerevisiae* strains, that are generally diploids, the majority of ale strains are tetraploid or more than diploid, with aneuploidies which hamper them to perfectly match the diploid or tetraploid status [23]. Aneuploidy and polyploidy, even if transient, can provide an adaptive advantage under selection, resulting in domestication [24,25]. A consequence of aneuploidy is the poor ability of ale yeasts to undergo sporulation. Sporulation ability is considered relevant for adapting strains to fluctuating and harsh environments, but it could be expensive in the nutrient-rich wort medium where most ale beer strains were isolated [21]. Continuous growth of ale yeasts in wort selects against this trait.

Another evidence for domestication is the ability *S. cerevisiae* ale strains to ferment maltotriose, which account for 20% of the total fermentable sugars in brewer's wort, but it is not normally present in high concentrations in natural yeast environments. During wort fermentation, yeast slowly consumes maltotriose only after glucose and maltose are depleted, and often maltotriose utilization remains incomplete. Maltose and maltotriose transporters are encoded by genes clustered in the subtelomeric *MAL* loci, which can be present on up to five different chromosomes depending upon the strain considered. A typical *MAL* (called *MALx*) locus includes a *MALT* (or *MALx1*) polysaccharide proton-symporter gene, a *MALS* (or *MALx2*) α-glucosidase gene which hydrolyses α-oligo-glucosides into glucose, and a *MALR* (also referred to *MALx3*) regulator gene that activates the transcription of *MALT* and *MALS* genes in the presence of maltose. While *MALS* genes are responsible for hydrolysis of both maltose and maltotriose, the *MALT* gene family comprises transporters with diverse substrate specificities. Generally, there are five known maltose-H^+ symporters in the *MAL* family [26]. The majority of *MALx1* transporter genes share high identity (>95%) each other and encode very specific high-affinity maltose transporters (Km ~ 2–5 mM), without any transport activity for maltotriose or other α-glucosides, including α-methylglucoside, palatinose, isomaltose, and melezitose [27,28]. The ale strains generally exhibited a remarkable expansion of copies of the *MAL3* locus, with the German beer strains which exhibited up to 15 copies

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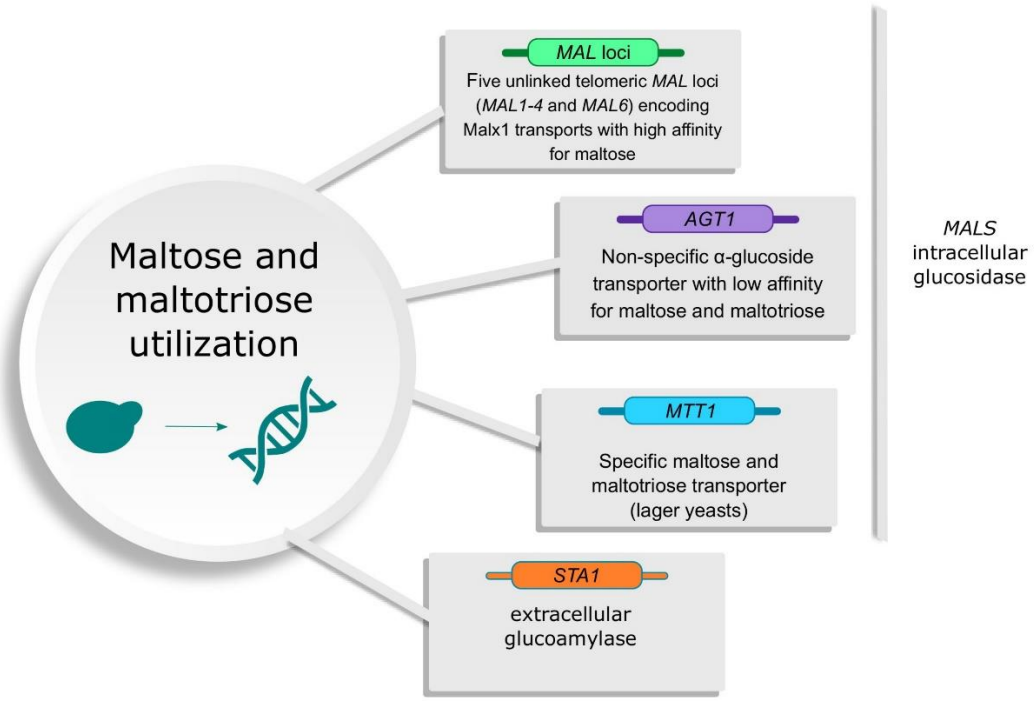
of *MAL31* gene [22] (**Figure 4**). In addition, all *S. cerevisiae* strains contain the *MAL1* locus at chromosome VII, which is considered the progenitor of other *MAL* loci. In ale strains *MAL11* gene at the *MAL1* locus is designed as *AGT1* and shares only 57% nucleotide identity with other *MALx1* transporter genes. *AGT1* gene encodes a complete 610 amino-acid long broad-substrate-specificity sugar-proton symporter that enables trehalose, sucrose (K_m ~ 8 mM) and maltotriose (K_m ~ 18.1 mM) uptake [29-31]. By contrast, in the majority of other *S. cerevisiae* strains *AGT1/MAL11* contains a premature stop codon at nucleotide 1183, which lead to loss-of-function. *AGT1* is present in several ale strains (clade 'Beer 1'), but absent and/or non-functional in the Wine and the 'Beer 2' clade [21] (**Figure 4**). Strains in the 'Beer 2' population utilize maltotriose efficiently, despite carrying unfunctional *AGT1/MAL11* [21]. This evidence suggests that alternative transporters are responsible for maltotriose uptake in these strains. Recently, Krogerus et al. [32] provided evidences that *STA1* gene encoding an extracellular glucoamylase could be involved in maltotriose utilization of 'Beer 2' strains during wort fermentation (**Figure 4**). Congruently, Ogata et al. [33] constructed a *S. cerevisiae* x *S. cerevisiae* hybrid capable to secrete Sta1 glucoamylase and to produce low-caloric beer by consuming almost all maltooligosaccharides present in wort.

Figure 4. Main maltose/maltotriose transporters in ale and lager yeasts.



Another example of the distinct genetic make-up in ale beer strains is the inability to produce 4-vinyl guaiacol (4VG), an unpleasant spicy clove-like compound. Genes *PAD1* and *FDC1* form a functional gene cluster at the end of chromosome IV and represent a detoxification system used by the cells against phenolic acids derived from barley [34,35]. Fdc1 decarboxylates ferulic acid into 4VG, while Pad1 provides a prenylated flavin-mononucleotide (FMN) cofactor of Fdc1p, required for its function. These genes were found functional in biofuel or non-industrial strains, but have different frameshift mutations or premature stop codons in beer yeasts [21], suggesting that domestication is frequently associated with the relaxation of some selective constraints on traits that are not advantageous in the specific industrial environment. Gonçalves et al. [22] found inactivation of *PAD1* and *FDC1* genes in *S. cerevisiae* strains used for German and British-style beers but not in lambic and most wheat beer strains.

The inactivation of aquaporin genes *AQY1* and *AQY2* represents another case of adaptive loss which occurred both in in wine and beer strains to increase fitness in environments with high osmolarity [22]. In particular the paralogs *AQY1* and *AQY2* genes encode water transporter and are

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involved in survival to freeze-thaw stress in wild strains from cold climates [36]. Passive water loss triggered by the high osmolarity conditions could be detrimental in strains which constantly experienced high sugar amount in surrounding medium. Congruently, the majority of ale yeasts showed frameshifting deletions or mutations giving rise to premature stop codons in aquaporin genes [22].

2.2. *Saccharomyces pastorianus*

Saccharomyces pastorianus, previously named by Hansen as *S. carlsbergensis* [37], is used worldwide for lager beer production. These bottom-fermenting yeasts are cold-tolerant alloaneuploid descendants of natural hybrids between the mesophilic *S. cerevisiae* species and a cryotolerant *Saccharomyces* non-*cerevisiae* parent. The parentage of these lager-brewing hybrids was a matter of dispute for decades [38], since several studies sustained the linkage between the non-*S. cerevisiae* parental strains and the genetically complex *Saccharomyces bayanus* species [38,39], a heterogeneous group of cold-tolerant strains, including the varieties *S. bayanus* and *Saccharomyces uvarum*. In 2011, Libkind and coworkers [40] firstly described the cryotolerant species *Saccharomyces eubayanus*, whose genome matched with the non-*S. cerevisiae*-type sub-genome of lager strains, apparently clarifying their parentage. *S. eubayanus* strains were originally discovered in Patagonia, Argentina, but later it was also isolated in North America [41,42], East Asia [43] and New Zealand [44]. In particular, Tibetan *S. eubayanus* strains showed higher identity with the non-*S. cerevisiae*-type sub-genome of lager hybrids than the Patagonian *S. eubayanus* strains, opening a further debate on the Asian origin of the *S. eubayanus* lager yeast parent [43].

Reconstruction of lager hybrid genomes showed that *S. pastorianus* arose approximately 500–600 years ago as a result of hybridization events directly influenced by social and cultural developments in human societies in Central Europe, during the Middle Ages. The most important anthropogenic intervention in the evolution of lager yeasts occurred in 1516 in Bavaria with the introduction of the Reinheitsgebot edict, the Beer Purity Law, which restricted the beer production to the winter months, between St Michael's Day (29 September) and St George's Day (23 April), insuring more stability and less bacterial contamination. At the same time, brewers in Bohemia tried to store beer in cool mountain caves, in order to improve the taste [1]. The consequent cooler temperature fermentation regime favored the *S. cerevisiae* x *S. eubayanus* interspecies hybrids over the parental populations. Several factors determined this ecological success: hybrids generally exhibit heterosis compared with one or both parents and combine the capability to utilize maltotriose of *S. cerevisiae* with the cold-tolerance of *S. eubayanus* [45]. Some researchers proposed that *S. eubayanus* initially was a wild yeast contaminant in the brewing process, with the selective advantage over the native ale yeasts to better grow at cooler temperatures [43]. However, *S. eubayanus* strains were isolated so far only in wild but not in brewing environments and never found in Europe.

After the initial hybridization events, differences in chromosomal organization [46] and genetic incompatibilities [47] led to genome reorganization by mitotic recombination, resulting in complex and highly heterogeneous lager genomes where loss of heterozygosity, chromosomal recombination and chromosome duplication were rampant events [40]. Compared to the complement of 32 chromosomes expected for an euploid *Saccharomyces* hybrid, *S. pastorianus* strains are highly aneuploid, containing 0 to 5 copies of each chromosome and only in few cases the canonical sets of two divergent *S. cerevisiae* and *S. eubayanus* orthologous chromosomes were retained [48,49]. As expected, mtDNA inheritance is uniparental in lager yeasts [50,51], with *S. eubayanus* being the main contributor of mitotype [52,53], even if sometimes recombinant haplotypes with introgression at the hotspot gene *COX2* were found [Peris et al., 2014]. Recently, Li et al. [54] found that the parent providing mtDNA in hybrids of *S. cerevisiae* and the cryotolerant species *S. uvarum* impacts temperature tolerance. Further, synthetic *S. cerevisiae* × *S. eubayanus* hybrids with *S. cerevisiae* mitotype were less cold-tolerant than isogenic hybrids with *S. eubayanus* mitotype [55].

Seminal studies based on transposon analysis and array- CGH data demonstrated that *S. pastorianus* strains divided into two distinct lineages corresponding to the geographical distribution of breweries: Saaz-type lager yeasts (hybrid Group I or *S. carlsbergensis*) exhibit a general triploid

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DNA content which have approximately haploid *S. cerevisiae* and diploid *S. eubayanus* chromosome complements; Frohberg-type (hybrid Group II) lager yeasts are generally tetraploid in DNA content with diploid *S. cerevisiae* and diploid S. *eubayanus* chromosome complements [56,57]. It was furthermore suggested that the *S. cerevisiae* parental genome was derived from ale yeast [57,58]. These lineages share many common properties, but they differ functionally in maltotriose utilization and cold tolerance. These functional differences correspond to genomic differences, since Saaz-type strains retained proportionally more DNA derived from *S. eubayanus* parent (that is unable to ferment maltotriose), explaining their cold-tolerance, while Frohberg strains contain approximately equal DNA content from *S. eubayanus* and *S. cerevisiae*, with a consequent higher ability to ferment maltotriose [58]. Accordingly, a comparative physiological study of 53 lager strains showed that Frohberg strains showed greater growth and superior fermentation compared to saaz-type and S. *eubayanus* strains all other strains, reaching 5% ABV in 3-4 days and maintaining highest viability values [59]. Performance of Saaz yeasts and *S. eubayanus* was limited by an inability to use wort maltotriose. Beers from Saaz fermentations were characterized by two- to six-fold lower production of the flavour compounds like methyl butanol, ethyl acetate and 3-methylbutyl acetate compared to Frohberg strains, rendering the latter more suitable in the actual beer industry [59].

The complete genome sequences of the Weihenstephan 34/70 strain, Frohberg-type lager yeast, and of *S. carlsbergensis* CBS 1513 (the first Saaz-type culture isolated by Emil Chr. Hansen in 1883) were released in 2009 and 2014, respectively [39,60]. Weihenstephan 34/70 (WS-34/70) has an allotetraploid genome containing 36 different chromosomes: 16 of *S. cerevisiae* (Scer) type, 12 of *S. eubayanus* (Seub) type and eight chimeric Scer/Seub chromosomes [39]. The *S. carlsbergensis* genome is 19.5-Mb long and consisted of 9 Scer, 26 Seub, and 7 chimeric Scer/Seub chromosomes [60]. After those projects, many other *S. pastorianus* genomes were made publicly available [53,61,62]. Comparative analyses showed that *S. pastorianus* Group (Saaz) I and II (Frohberg) genomes exhibit nine lager-specific genes at the subtelomeric regions [63]. These sub-telomeric regions are enriched for genes with functions determining the cell's interaction with its environment, such as nutrient uptake, sugar utilization, inhibitor tolerance and flocculation. Furthermore, four rearrangements between *S. cerevisiae* and *S. eubayanus* sub-genomes were found at loci *ZUO1*, *HSP82*, *XRN1*/*KEM1* and *MAT*, leading to chimeric chromosomes. These breakpoints are identical between Group I and II *S. pastorianus* strains suggesting that they share a common *S. cerevisiae* x *S. eubayanus* hybrid ancestor, and that the differences between Group 1 and Group 2 strains emerged subsequently [60,61]. In particular, Group 2 strains possess more heterozygous *S. cerevisiae* regions than Group 1 strains. These allelic variants in Group 2 strains consisted of sequences similar to those found in Group 1 and of sequences of a different *S. cerevisiae* genome [53]. Recently Nanopore sequencing of the *S. pastorianus* Frohberg-type strain CBS 1483 resolved bias in assemblies of chimeric genomes at subtelomeric regions and demonstrated that Saaz- and Frohberg-type strains originated from a single hybridization involving an ancestral heterozygous *S. cerevisiae* strain, followed by different evolutionary trajectories [64].

While several advances were taken on genomic structures of *S. pastorianus*, molecular effectors of several industrially relevant phenotypes and their evolutionary origin remain unknown. For instance, *S. pastorianus* inherited *MAL* genes from both *S. cerevisiae* and *S. eubayanus*, but the *AGT1* gene responsible for maltotriose uptake in ale yeasts (*ScAGT1*) is cold sensitive and prematurely truncated in *S. pastorianus*. By contrast, the *S. eubayanus* homologue *AGT1* gene (*SeAGT1*) shows only 85% identity at amino-acid level with ScAgt1 and encodes a cold-tolerant α-glucoside transporter with similar affinities for maltose and maltotriose (Km ~ 17 and 22 mM, respectively). Another gene involved in sugar uptake both in *S. pastorianus* and baker's/distiller's yeasts is *MTT1*, also called *MTY1*, encoding a H^+-symport specific for maltose, maltotriose, trehalose, turanose, and especially for maltotriose (Km of 16–27 mM for maltotriose and 61–88 mM for maltose [28,31,65]. Mtt1 functions better at lower temperatures than Agt1, explaining the adaptation of lager strains to cold fermentation conditions (**Figure 4**). *MTT1* genes change in copy number in a strain-dependent fashion and lager strains which exhibit multiple copies of *MTT1* enhance their maltotriose fermentation capacity [66]. Interestingly, *MTT1* gene is located on *S. cerevisiae* ChrVII, but shares only

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90% identity with *MALx1* and 54% with *ScAGT1* genes. Several domains in *S. pastorianus* Mtt1 protein had high similarity to maltose transporters from *S. eubayanus*, suggesting that Mtt1 is more related to *S. eubayanus* than *S. cerevisiae* ortholog. Recently, evolutionary studies showed that recombination of different *SeMALx1* genes yielded chimeric, neo-functionalized genes that encoded maltotriose transporters similar to Mtt1 [67,68]. Paradoxically, Tibetan *S. eubayanus* strains, that most close to the putative cold-tolerant parent of *S. pastorianus*, were unable to use maltose and maltotriose, due to a nonsynonymous mutation in *SeMALR1* which hampered the expression of *SeMALT* genes [69].

3. Mimic lager yeasts by artificial hybridization

In addition to *S. pastorianus*, other hybrids have been isolated in brewing environment, such as hybrids between *S. cerevisiae* and *Saccharomyces kudriavzevii* used for the fermentation of several Belgian Trappist beers [70] or *Saccharomyces bayanus* (*S. eubayanus* × *S. uvarum*) hybrids isolated as contaminants from beer [38,71]. Taking these natural hybrids as templates, novel synthetic interspecific hybrids have been constructed in laboratory to combine desired phenotypes in a single clone. Compared to parents, interspecies hybrids often show interesting traits which make them attractive for fermentative purposes, such as the synergistic phenomenon of heterosis, also called hybrid vigor, that is the tendency to outperform parents in fermentative performance; enhanced homeostasis (also called canalization or robustness), consisting in the ability of organisms to buffer the effects of external perturbations through metabolic, physiological or developmental adjustments, in order to maintain fitness in various habitats; phenotypic novelty and additivity and mid-parent phenotypes (semidominance) for some traits [72]. The approaches to perform sexual hybridization can be different, namely mass-mating, rare-mating and spore-to-spore mating [73]. In general, the first step is the sporulation of the yeast with the generation of gametes; then the spores can merge in a zygote after being randomly shuffled (mass-mating) or after being physically placed in contact (spore-to-spore mating) or even after a fortuitous homozygosis (rare-mating). The success of these techniques is strictly affected by reproductive isolation [74,75], so the parental strains are selected among the same genus in order to maximize the results.

De novo S. cerevisiae × *S. eubayanus* hybrids were successfully constructed for lager beer production [45,76,77,78,79,80,81]. In these hybrids parental sub-genome interactions resulted in several positive traits, such as cryotolerance, maltotriose utilization and strong flocculation. Hybrids also exhibited a broader temperature tolerance than their parental strains [80] and fermented faster, producing beer with higher alcohol content than the parents. Hybrids can lead to beers with complex and enriched aromatic profile. Moreover, hybrids with different ploidy show different aroma profile, indeed tetraploid hybrids exhibit the higher concentrations of ethyl and acetate esters than triploid hybrids, that contains proportionally more S. cerevisiae sub-genome and consecutively higher gene copy numbers and transcription levels [77] However, the majority of *de novo S. cerevisiae* × *S. eubayanus* hybrids also produced beer with smoky flavors typically associated with the presence of 4VG. This sensorial attribute, also called "phenolic off flavor" (POF) is often negatively perceived in the lager beer industry. The majority of wild *S. cerevisiae* strains and all known *S. eubayanus* strains characterized so far exhibit POF^+ phenotype. Three strategies were successfully adopted to circumvent this detrimental trait. Krogerus et al. [77] used rare mating to obtain allotetraploids which undergone meiosis in order to select POF^- segregants. Rare mating events were tracked by using complementary auxotrophic derivatives of wild strains. Alternatively, Diderich et al. [82] exploited UV mutagenesis to select POF^- *S. eubayanus* mutants that were crossed with a POF^+ *S. cerevisiae* parental strain. POF^- phenotype was selectable as unfunctional *PAD1*/*FDC1* genes reduce the cell ability to grow in presence of ferulic acid. Finally, CRISPR/Cas system was harnessed to produce cisgenic POF^- variants of lager yeasts, as well as to generate *de novo* POF^- interspecific hybrids by introducing a naturally occurring loss-of-function mutation in the *FDC1* gene [83]. Although this last approach can potentially revolutionize the genome editing of beer yeasts, recently, organisms modified by the CRISPR-Cas technique have been included in the GMO classification by EU legislation, hampering their usage in food chain supply [84].

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Hybrids alternative to *S. cerevisiae* x *S. eubayanus* were also proposed to combine cold- and sugar tolerance. Cold-tolerant *Saccharomyces* species including *Saccharomyces arboricola*, *Saccharomyces mikatae* and *Saccharomyces uvarum* were used as surrogates of *S. eubayanus* in crosses with *S. cerevisiae* [85,86]. Sato et al. [85] performed mass mating between top-fermenting *S. cerevisiae* yeasts and a cryotolerant *S. uvarum* strain and selected hybrid candidates by combining the *S. uvarum* contribution for melibiose assimilation with the *S. cerevisiae* contribution for growth ability at 35°C. The resulting *S. cerevisiae* x *S. uvarum* hybrids outperformed *S. cerevisiae* top-fermenting parents in fermentation vigor, resembling the bottom-fermenting control strains. Nikulin and co-workers [86] expanded the range of cryotolerant parental strains, including *S. arboricola* and *S. mikatae* as *Saccharomyces* non-*cerevisiae* counterpart in hybridization cross. Although rare mating technique should give allotetraploid hybrids, hybrids with variable ploidies (from 2 to 4n) were obtained. Hybrids with higher ploidy level (4n and 3n) showed higher fermentative vigor than 2n hybrids, that probably resulted from fortuitous spore-to-spore mating. Interestingly, *S. arboricola*- and *S. mikatae*-derived hybrids performed well in wort, despite the parent strains not demonstrating any clear capabilities of utilizing maltose or maltotriose. All hybrids increased formation of desirable aroma-active esters, such as 3-methylbutyl acetate (banana aroma), ethyl hexanoate (apple aroma) and ethyl octanoate (fruity aroma). However, like *S. cerevisiae* x *S. eubayanus* hybrids, all these alternative hybrids exhibited POF^+ phenotype.

4. Evolutionary Engineering

Evolutionary engineering techniques have been extensively used to improve yeast cell phenotypes in wine and sake fermentation and recently were also adopted in brewing to improve sugar utilization [87], flavor profile [88], and stress tolerance [89-91]. For instance, residual amounts of maltotriose are detrimental for breweries as it increases the probability of beer spoilage and lead to quality and economic problems. Maltotriose uptake and utilization were enhanced in *S. pastorianus* strain CBS 1483 by continuous cultivation on a maltotriose-enriched sugar mixture [87]. As result, evolved derivatives of strain CBS 1483 exhibited lower residual maltotriose and higher ethanol concentration than the parental strain. Similarly, Blieck et al. [89] isolated two variants of a lager yeast strain with improved fermentation performance after successive fermentations with UV-treated yeasts in very high-gravity wort (> 22 °P). Huuskonen et al. [91] treated brewing yeast cells with ethyl methanesulfonate (EMS) and exposed the mutagenized cells to conditions typical for the final stages of very high-gravity fermentations (high ethanol concentration and maltose and maltotriose as the sole fermentable sugars). Yeast variants with the capability to survive these conditions exhibited improved fermentation performance in very high-gravity (24 °P) wort, mainly at the end of fermentation, when conditions resembled those under which the variants were selected. Yu et al. [92] used a similar approach to improve high gravity fermentative performance of beer yeast treated with both EMS and UV mutagenesis. Ekberg and co-workers [90] repetitively cultured lager EMS-mutagenized yeast in the presence of high sorbitol amount in order to select brewer's yeast variants exhibiting faster and more complete brewer's wort fermentative performance. More recently, genetic instability of *de novo S. cerevisiae* x *S. eubayanus* hybrids was exploited by cultivation under high ethanol concentration to gain high-ethanol tolerant derivatives for lager style beer production [79].

While the adaptive evolutionary approaches described above rely on the alteration of phenotypes with a direct adaptive advantage with regard to yeast survival or growth, other evolutionary engineering strategies are “directionless” in the sense that they rely on the usage of drugs and analog compounds, which are not directly related to the increase in desired phenotype. Gibson et al. [88] exposed repeatedly lager strain to sub-lethal level of chlorsulfuron, in order to gain derivatives with reduced diacetyl production compared to the wild strain. Diacetyl is responsible for unpleasure buttery flavor in lager-style beer and resulted from α-acetolactate by spontaneous decarboxylation. Chlorsulfuron inhibits the acetohydroxy acid synthase Ilv2 responsible for α-acetolactate production from pyruvate. Tolerance to chlorsulfuron may result in higher or lower

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diacetyl production as this phenotype is not expected to have a direct impact on the survival or fermentation performance of the strain. Similar "directionless" approaches were used to improve flavor profiles in sake yeasts (**Table 1**) and entailed the accurate screening of evolved strains before their industrial exploitation.

Table 1. "Directionless" evolutionary engineering approaches in improvement of flavor-related phenotypes.

Compounds	Secondary metabolites	Flavor impact	References
5,5,5-trifluoro DL-leucine	Increase in 3-methylbutyl acetate	banana/pear aroma	[93]
isoamyl monofluoroacetate	Increase in 3-methylbutyl acetate	banana/pear aroma	[94]
1-farnesylpyridinium	Increase in 3-methylbutyl acetate	banana/pear aroma	[95]
chlorsulfuron	decrease in diacetyl	buttery aroma	[88]
cerulenin	Increase in ethyl caproate	apple aroma	[96]
fluoro-DL-phenylalanine	Increase in phenylethyl acetate	rose aroma	[97]

5. Fermented Food as Reservoir of Novel *S. cerevisiae* Brewing Starters

In recent years, several studies highlighted the potential of feral *S. cerevisiae* strains isolated from spontaneously fermented beers or from alternative food matrices, for the production of beers with novel flavor profiles and other desirable properties. Yeast isolation represents one of the most interesting solutions for brewers, since it takes advantage from the natural biodiversity of the microorganisms adapted to grow in their habitats. On the other hands, knowledge on molecular mechanisms underpinning some relevant beer-related traits in ale and lager yeasts have been highly improved in recent years. The charting of these genotyping-phenotype correlation maps recently can assist the accurate and marker assisted selection of natural variants with the highest aptitude for brewing at least partially avoiding time-consuming trials-and-error procedures (**Figure 3**).

For instance, Brazilian Cachaça spirit beverages are high-ethanol niches where *S. cerevisiae* strains were isolated with a brewing aptitude comparable with commercial beer strains with regards to ethanol yields and cellular viability [98]. *S. cerevisiae* strains were isolated from traditional Beninese sorghum beer (*Tchoukoutou*) and tested in 12 °P sorghum wort fermentation using ale yeast TUM68 as control [99]. These indigenous yeasts were more aromatogenic than the commercial ale strain as they were able to produce higher alcohols from sorghum wort at 27 °C, even if this wort generally contained low amounts of branched amino acids. Some wine yeasts, like DBVPG 1058 isolated from grape must, were exploitable in brewing as they produced ethanol yield comparable with commercial beer strains, as well as acetaldehyde, diacetyl and other off-flavor compounds at lower concentrations than the sensory thresholds [100]. However, the majority of wine strains generally exhibited poor fermentative vigor in brewer's wort and were more adapted to re-fermentation process in bottle [101].

Although *S. cerevisiae* yeasts from various alcoholic beverages, such as pulche, tequila or sake, were proposed to have brewing potential [102], only baker yeasts were experimentally demonstrated to be truly exploitable in wort fermentation. This is historically proven by old style beers such as the Russian Kvass or the Finland's sahti beers which are still brewed by natural fermentation of bread or by using baker's yeasts, respectively [103,104]. Remarkably, beer and baker's yeasts are phylogenetically closed [21] and grow on maltotriose as carbon source even under anaerobic conditions [105]. Most of the *S. cerevisiae* strains isolated from sourdough were able to ferment glucose, maltose and trehalose. Interestingly, the trehalose uptake is carried out by the same transporters which uptake maltose and maltoriose, rendering these strains suitable to ferment wort [106,107]. Gonçalves and co-workers [22] observed that, like beer strains, bread strains were enriched in *MAL3x* copies and in *IMA1* gene copies, which encodes a major isomaltase. These evidences

suggested that bread and beer strains could share similar aptitude for maltose and maltotriose utilization. Marongiu et al. [106] demonstrated that strain S38 isolated from Sardinian sourdough produced beer with a chemical and sensory profile similar to that obtained with the brewer's strain Safbrew-F2. Durum wheat beer was usefully produced by using a *S. cerevisiae* yeast isolated from sourdough, which overcame the commercial brewing yeast in ethanol content, lowering the pH and production of the higher content of esters and alcohols. More recently, sourdough back-slopping was used in wort fermentation to produce acidic beer by the action of both yeasts and lactic acid bacteria populations [108].

Potential drawbacks in brewing utilization of sourdough yeasts are that baker's yeasts did not exhibit flocculation trait required for brewing [109] and they generally possess functional *PAD1* and *FDC1* responsible for the POF^+ phenotype. This limits the exploitation of sourdough strains for brewing beer specialties, such as wheat beers, lambic beers and some ale craft beers. However, Peter et al. [23] found 8 out of 32 analyzed bakery strains carrying homozygous nonsense or frameshift mutations on *FDC1* or *PAD1*, suggesting that baker's *S. cerevisiae* biodiversity is still unexplored and that sourdough ecosystems could be reservoirs of naturally POF^- individuals.

6. Non-*Saccharomyces* yeasts

Recent trends are focused on the personalization of the starter culture for the wort fermentation by the exploitation of non-*Saccharomyces* yeasts, or non-conventional yeasts. These yeasts have been conventionally considered detrimental for the final products as they negatively impact some sensorial properties of the fermented alcoholic beverages, such as the turbidity, viscosity or mouthfeel [110,111,112,113,114]. However, appropriate strain selection and accurate management of brewing parameters can lead to novel beers with alternative aromatic tastes. Non-*Saccharomyces* yeasts, therefore, can be used for bio-flavoring, a process that can fulfill the modern consumer's expectations to receive a product with enhanced aroma profile without chemical additives. Although the application of non-conventional yeasts has been extensively used in wine-making to increase flavor-active compounds [115-117], only in recent years some studies tried to apply them to the brewing process [113,118,119]. Compared to *Saccharomyces*, these yeasts generally resulted to have a lower ethanol yield, so they are rather used in co-fermentation or in sequential fermentation with classical *Saccharomyces* brewing yeasts then as a pure starter culture. Otherwise, this low ethanol production can lead to low-alcoholic (0.5–1.2% v/v) or even alcohol-free (<0.5% v/v) beers, which are an increasingly demanded beverages [120]. For instance, *Saccharomycodes ludwigii* [121] and *Pichia kluyveri* [122] were successfully used to produce alcohol-free beers with rich flavor profiles owing to its aroma-related compounds production and low performance in fermenting maltose and maltotriose. Similarly, *Zygosaccharomyces rouxii* was suitable for producing low-alcohol beers as it consumed ethanol under aerobic conditions and produced actively desired flavor compounds [123].

The most investigated non-conventional yeast for brewing purpose belonged to *Brettanomyces/Dekkera* genera (**Table 2**). Taxonomically, *Dekkera* genus includes two species, namely *D. bruxellensis* and *Dekkera anomala*, which describe the teleomorphic (sexual) state of the anamorphs *Brettanomyces bruxellensis* and *Brettanomyces anomalus*. Practically, the terms *"Brettanomyces" and "Dekkera"* are used as synonyms. *Brettanomyces* yeast was the first patented microorganism (UK patent GB190328184) in history for the manufacture of English beers such as ale, stout and porter beers [110]. Like S. *cerevisiae*, *B./D. bruxellensis* and *B./D. anomalus* are facultative anaerobes and Crabtree-positive species, but differently from *S. cerevisiae* they are also capable of producing, accumulating and later consuming high concentrations of acetic acid in aerobic conditions. These species are well known to the beverage industry as a spoilage yeasts in wine and soft drinks where they are also responsible for the so called "Brett flavor". "Brett flavor" is complex sensory profile referring to negative attributes, like "leather", "manure" or "horse sweat" flavor, but also to overall fruity or floral characters. The most relevant molecules released by *B./D. bruxellensis* and *B./D. anomalus* and contributing to "Brett flavor" are POF compounds (such as 4-ethylguaiacol, 4-ethylphenol, 4-ethylcatechol and their pre-cursors 4VG, 4-vinylphenol and 4-vinylcatechol), substituted tetrahydropyridines (including 2-ethyltetrahydropyridine, 2-acetyltetrahydropyridine,

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and 2-acetylpyrroline), and volatile esters [110]. In addition to wine and soft drinks spoilage, *B./D. bruxellensis and B./D. anomalus* can be found in mixed fermentations of gueuze and lambic beers. Most *B./D. bruxellensis and B./D. anomalus* strains can ferment the main sugars present (**Table 2**). As a result of β-glucosidase activity, *B./D. anomalus* can hydrolyze glucoside-bound monoterpenes, which are present in many fruits and also in brewers' wort that comes from hops [124]. The breakdown of these bonds releases monoterpenes changes them into active flavor compounds. This could increase or modify the hop aroma because many of the released monoterpenes, such as linalool, are the key aroma substances from hops [125].

In addition to *Brettanomyces/Dekkera* yeasts, other genera and species have been considered for brewing, such as *Schizosaccharomyces pombe*, *Lachancea thermotolerans*, *Wickerhamomyces anomalus*, *Torulaspora delbrueckii* and *Zygotorulaspora florentina*. For example, *T. delbrueckii* was traditionally used in the production of Bavarian wheat beers (Hefeweizen) [126]. This yeast can grow in the presence of up to 90 ppm iso*α*-acids in the medium, a concentration that correlates to highly hopped beer styles [127]. Compared to *S. cerevisiae* monoculture, co-culture of *S. cerevisiae* and *T. delbrueckii* in 1:20 ratio appeared to be more promising for fusel alcohol, ethyl decanoate and ethyl dodecanoate production, leading to specialty beer with flavor distinct from conventional ales [114]. Callejo and co-worker [128] reported that *S. pombe* produced higher alcohol content and enhanced foam consistency and persistence better than *T. delbrueckii, L. thermotolerans* and *S. ludwigii*, whilst *T. delbrueckii* produce highly fruity beer Domizio et al. [129] suggested the usage of pure culture of *L. thermotolerans* in sour beer production since this non-conventional yeast lowered the pH better than *S. cerevisiae*. Another promising non-*Saccharomyces* yeast for brewing is *W. anomalus*, a species frequently associated to a range of cereal-based sources. Mixed fermentation with lager yeast WS34/70 and *W. anomalus* CBS 261 in a 1:1 ratio enhanced the amounts of hexadecanoate, isoamyl alcohol and 2-phenyl ethanol compared to control conditions, improving the fruity flavor perception in the final product [130].

Table 2. This is a table. Tables should be placed in the main text near to the first time they are cited.

Yeast	Strain	Fermentation conditions	Reference
Blastobotrys mokoenaii	X9113	pure	[118]
Brettanomyces anomalus	X9073	pure/sequentially inoculated with Ale 514 brewing yeast	[118]
Brettanomyces bruxellensis	CBS 3025, AWRI1499	pure/sequentially inoculated with Ale 514 brewing yeast	[118]
Brettanomyces naardenensis	NRRL Y-5740	pure/sequentially inoculated with Ale 514 brewing yeast	[118]
Candida stellata	X9023	pure	[118]
Citeromyces matritensis	ST1312/081	pure	[118]
Debaryomyces hansenii	x38	pure	[118]
Kodamaea ohmeri	x22	pure	[118]
Lachancea thermotolerans	DiSVA 322	pure/co-colture with S. cerevisiae starter strain US-05	[131]
Lachancea thermotolerans	x9005	pure	[118]
Metschnikowia reukaufi	Y6.3K/FT11 B	pure	[118]
Pichia anomala	x9015, x10	pure/sequentially inoculated with Ale 514 brewing yeast	[118]
Pichia kluyverii	x21, x36	pure/sequentially inoculated with Ale 514 brewing yeast	[118]
Pichia kudriavzevii	x12, X9035	pure/sequentially inoculated with Ale 514 brewing yeast	[118]

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Saccharomycodes ludwigii	DBVPG 3010, DBVPG 3304, DBVPG 3398, DBVPG 3931, DBVPG 4116, DBVPG 6721	pure	[120]
Starmerella bacillaris	X9029	pure	[118]
Starmerella bombicola	V10.2Y A1	pure	[118]
Torulaspora delbrueckii	DiSVA 254	pure/co-colture with S. cerevisiae starter strain US-05	[131]
Torulaspora delbrueckii	ST1312/167	pure/sequentially inoculated with Ale 514 brewing yeast	[118]
Wickerhamomyces anomalus	DiSVA 2	pure/co-colture with S. cerevisiae starter strain US-05	[132]
Zygosaccharomyces rouxii	DBVPG 4084, DBVPG 6187, DBVPG 6424, DBVPG 6463, DBVPG 6921	pure	[120]
Zygotorulaspora florentina	DiSVA 263	pure/co-colture with S. cerevisiae starter strain US-05	[132]
Zygotorulaspora florentina	X9022	pure/sequentially inoculated with Ale 514 brewing yeast	[118]

5. Conclusions

This study provided an overview of the main non-genetic engineering techniques used so far to meet the challenging requests for brewing yeasts diversification arisen from the emerging craft beer market. Synthetic *S. cerevisiae* x *Saccharomyces* non-*cerevisiae* hybrids, non-conventional yeasts and *S. cerevisiae* natural variants from alternative bio-reservoirs represent the most promising frontiers for craft brewing, as they impact and significantly enrich the aroma profile of the final products. We also provided evidences on how novel discoveries on genomic signatures of brewing relevant phenotypes can further steer and enhance the process of innovation in beer starter culture selection. Additional improvements of these novel brewing yeasts can be reached by exploiting evolutionary strategy approaches or, alternatively, by using combined strategies where two of these techniques were jointed.

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