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

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Received: 21 November 2019; Accepted: 31 December 2019; Published: 9 January 2020

Abstract: Beer is a fermented beverage with a history as old as human civilization. Ales and lagers are by far the most common beers; however, diversification is becoming increasingly important in the brewing market and the brewers are continuously interested in improving and extending the range of products, especially in the craft brewery sector. Fermentation is one of the widest spaces for innovation in the brewing process. Besides \textit{Saccharomyces cerevisiae} ale and \textit{Saccharomyces pastorianus} lager strains conventionally used in macro-breweries, there is an increasing demand for novel yeast starter cultures tailored for producing beer styles with diversified aroma profiles. Recently, four genetic engineering-free approaches expanded the genetic background and the phenotypic biodiversity of brewing yeasts and allowed novel costumed-designed starter cultures to be developed: (1) the research for new performant \textit{S. cerevisiae} yeasts from fermented foods alternative to beer; (2) the creation of synthetic hybrids between \textit{S. cerevisiae} and \textit{Saccharomyces non-cerevisiae} in order to mimic lager yeasts; (3) the exploitation of evolutionary engineering approaches; (4) the usage of non-\textit{Saccharomyces} yeasts. Here, we summarized the 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. The resulting correlation maps between genotypes and relevant brewing phenotypes can assist and further improve the search for novel craft beer starter yeasts, enhancing the portfolio of diversified products offered to the final customer.

Keywords: craft brewing; \textit{Saccharomyces cerevisiae}; \textit{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]. Nowadays, the 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 productive process, beer appears to be a highly consolidated and sufficiently known product. This consideration is, however, disproved thinking of all the sciences behind the brewing process: Microbiology, chemistry, agronomy, but even logistic, marketing, process engineering, and health science cooperate to obtain high-quality and versatile products competitive on the market (Figure 1).

Conventionally, the term “beer” refers to 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 \textit{Saccharomyces} yeasts. Ale, lager, porter, stout, lambic, waisse, and many other words can be found beside the general “beer” to indicate specific beer products with peculiar visive and sensorial and chemical-compositional properties, such as bitterness, alcohol-by-volume...
content, as well as original and final gravity [4]. However, the principal separation criterion accepted for beer classification relies on the type of brewing process, which allows the separation of beers in three macro-categories, such as ale, lager, and lambic. Ale beers are brewed by top-fermenting *Saccharomyces cerevisiae* strains at fermentation a temperature of 15 °C–25 °C, while lager-style beer involves allopolyploid *Saccharomyces pastorianus* yeasts in a process conducted at a temperature of 8 °C–12 °C [5]. Finally, lambic-style beer is obtained by a spontaneous fermentation because, originally, it was performed by just exposing the wort to the air letting it become colonized by wild yeasts and bacteria. Apart from some 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 toward the mild lager beer styles. These products represent 90% of the beer market [6].

Starting from the 1980s, an increasing trend in food and beverage industries 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 toward increased demand for healthier food and drinks [8]. In agreement with these trends, beer consumption decreased in Northern America and Europe. In this highly competitive scenario, segmentation of the beer market provided an avenue for businesses to remain viable and craft beer rapidly increased in popularity in Northern America and Europe. [9]. High-income and sophisticated consumers looked for a variety of local beer products with high-quality ingredients and a high level of “beverage culture” [10,11]. In the US, the craft market grew from 5.7% to 12.3% 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 [15], they exhibit some common aspects. Generally, craft beer is produced by small, independent, and traditional breweries [16] and

![Figure 1. Multi-disciplinary perspective for challenging the brewing complexity. Basic ingredients required in the brewing process are summarized in the middle, while a plethora of disciplines involved in brewing science are depicted on the outside.](image_url)
it is usually an unfiltered, unpasteurized beverage, without additional nitrogen or carbon dioxide pressure and re-fermented in the bottle. Alternative ingredients such as tobacco, tomatoes, coffee, cacao, fruit, and a range of spices are frequently used [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].

Different brewing systems and beer styles require different yeast starter cultures. Fermentation plays a key role in determining flavorful alternative products, as yeast metabolism strongly affects not only alcohol yield from maltose and maltotriose, but also flavor and aroma composition. Pyruvate produced by yeast glycolysis 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 represents the widest space for beer diversification within 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 draw maps between genome landscape and industrially interesting phenotypes. This review summarizes the main knowledge of beer yeast genomics and describes how this information can drive and accelerate the selection of novel yeast starters for brewing. Four main innovation trends were delineated to expand the portfolio of craft brewing starters, including: (i) the mimicking of lager yeasts by the creation of synthetic hybrids between \textit{S. cerevisiae} and cold-tolerant \textit{Saccharomyces non-cerevisiae} strains; (ii) the evolutionary engineering techniques to improve fermentative performance in high-brevity wort and to enhance flavor; (iii) the search of new performant \textit{S. cerevisiae} yeasts from alternative bioreservoirs such as artisanal fermented food; (iv) the usage of non-\textit{S. cerevisiae} yeasts as flavoring agents (Figure 2).

![Brewing yeast development strategies](image)

**Figure 2.** Main trends in brewing innovation. Knowledge on molecular mechanisms underpinning beer-relevant phenotypes drives the search for novel and personalized starter cultures by the application of non-genetic engineering techniques.
2. Brewing Yeasts Through the Lens of Genomics

The role of 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 beer production, pioneered by Christian Hansen, certainly improved the consistency of quality beer, but 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 usage in ale and lager beer production. Ale yeasts, classified as top-fermenters, carry out fermentation at relatively high temperatures (15–26 °C) and tend to float to the top of the vat at the end of fermentation. Cold-tolerant lager yeasts ferment at lower temperatures (8–15 °C) and sediment to the bottom of the fermentation vessel, thus they are recognized as bottom-fermenters. Recent advances in next-generation sequencing technologies boosted the number of analyzed ale and lager genomes, while the developments of phenomics approaches made it possible to analyze many phenotypic traits under the same comparative frameshift. Overall these efforts strongly contribute to link genes to their related industrial phenotypes and provide new insights on how brewing yeasts evolved, revealing the main domestication events which made ale and lager yeasts differentially adapted to specific industrial niches. This body of knowledge can be useful in understanding the genetic signatures of brewing traits and, in turn, to implement the marker-assisted selection of novel brewing starter cultures (Figure 3).

2.1. Saccharomyces cerevisiae Ale Yeasts

Comparative genomics demonstrated that most of the S. cerevisiae ale 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, ale strains exhibit large-scale variations in genome structure, including changes in ploidy and large segmental duplications or copy number variations. Most of the small structural genome variations are commonly located in telomeric and sub-telomeric regions, which represent typically hotspots for evolution. Unlike wild S. cerevisiae strains, that are generally diploids, most ale strains are tetraploid or more than diploid, with aneuploidies that hamper them to perfectly match the diploid or tetraploid status [23]. Aneuploidy and polyploidy, even if transient, can provide an adaptive advantage under selection [24,25], but make ale yeasts poorly able to sporulate. 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 of ale strains to ferment maltotriose, which accounts for 20% of the total fermentable sugars in brewer’s wort, but 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 hydrolyzes α-oligo-glucosides into glucose, and a MALR (also referred to MALx3) regulator gene that activates the transcription of MALT and MALS genes in presence of maltose. While MALS genes are responsible for the 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 a high identity (>95%) with 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 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 the majority of ale strains (clade ‘Beer 1’) [21], the MAL11 gene at the MAL1 locus is designed as AGT1 and shares only 57% nucleotide identity with other MALx1 transporter genes. The AGT1 gene encodes a complete 610 amino-acid long broad-substrate-specificity sugar-proton-symporter that enables trehalose, sucrose ($K_m \approx 8$ mM), and maltotriose ($K_m \approx 18.1$ mM) uptake [29–31]. By contrast, in other S. cerevisiae strains, such as wine strains and strains from ‘Beer 2’ clade, AGT1/MAL11 contains a premature stop codon at nucleotide 1183, which leads to loss-of-function (Figure 4). Despite this unfunctional AGT1/MAL11 variant, strains in the ‘Beer 2’ clade utilize maltotriose efficiently, suggesting that alternative transporters are responsible for maltotriose uptake [21]. Recently, Krogerus et al. [32] provided evidence that the glucoamylase Sta1 extracellularly hydrolyzed maltotriose in these ‘Beer 2’ strains, contributing to complete wort sugar consumption (Figure 4). Congruently, Ogata et al. [33] constructed a S. cerevisiae × S. cerevisiae hybrid capable to secrete Sta1 glucoamylase and to produce low-caloric beer by consuming almost all maltooligosaccharides present in wort.

**Figure 3.** Marker-assisted selection of natural variants harboring brewing relevant traits. Clonal selection provides the best adapted candidates for brewing, while genomics and phenomics outline the map of loci responsible for brewing characteristics. This information, in turn, can be used to improve the selection of novel powerful and costumed-designed candidates. Letters on the columns represent statistical significance.
Another example supporting the distinct genetic make-up of ale beer strains is the inability to produce 4-vinyl guaiacol (4 VG), 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 4 VG, while Pad1 provides the prenylated flavin-mononucleotide (FMN) cofactor of Fdc1, required for its function. These genes are functional in biofuel or non-industrial strains, but have different frameshift mutations or premature stop codons in beer strains [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-of-function, which occurred both in wine and beer strains [22]. In particular, the AQY1 and AQY2 paralogs encode water transporters involved in the survival of wild strains from cold climates to freeze-thaw stress [36]. Passive water loss triggered by the high osmolarity conditions could be detrimental in strains that constantly experienced high sugar amounts in the 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 co-workers [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* was originally discovered in Patagonia, but later it was also isolated in North America [41,42], East Asia [43], and New Zealand [44]. 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 × S. eubayanus* interspecies hybrids over the parental populations. 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 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 the wild but not in brewing environments and never found in Europe.

After the initial hybridization events, differences in the chromosomal organization [46] and genetic incompatibilities [47] between parental haplotypes triggered an extensive genome reorganization where the loss of heterozygosity, chromosomal recombination, and chromosome duplication were rampant events [40]. Compared to the complement of 32 chromosomes expected for a 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 [41]. Recently, Li et al. [54] found that the parent providing mtDNA impacts temperature tolerance in hybrids of *S. cerevisiae* and the cryotolerant species *S. warau*. Further, synthetic *S. cerevisiae × S. eubayanus* hybrids with *S. cerevisiae* mitotype were less cold-tolerant than isogenic hybrids with *S. eubayanus* mitotype, indicating that mitotype is a selectable brewing trait in artificial hybrid creation [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 DNA content, which has 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 yeasts [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 Saaz yeasts and *S. eubayanus* strains had poor ability to use wort maltotriose; consequently, Frohberg strains showed greater growth and a superior fermentation rate compared to Saaz-type and *S. eubayanus* strains [59]. Beers achieved with from
Saaz-type strains showed by two- to six-fold lower production of the flavor compounds 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 these projects, many other S. pastorianus genomes were released [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 in genes involved in nutrient uptake, sugar utilization, 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 × 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 Scer 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 genomic structures of S. pastorianus have been extensively studied, molecular effectors of several industrially relevant phenotypes remain poorly known. 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 the amino-acid level with ScAgT1 and encodes a cold-tolerant α-glucoside transporter with similar affinities for maltose and maltotriose (K_m ~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 (K_m 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 that exhibit multiple copies of MTT1, which enhance their maltotriose fermentation capacity [66]. Interestingly, the MTT1 gene is located on S. cerevisiae ChrVII, but is more related to S. eubayanus than to S. cerevisiae orthologs. Recent evolutionary studies showed that recombination among different SeMALx1 genes yielded chimeric, neo-functionalized genes that encoded maltotriose transporters similar to Mtt1 [67,68]. Paradoxically, Tibetan S. eubayanus strains, which are the closest relatives to the putative cold-tolerant parent of S. pastorianus, were unable to use maltose and maltotriose, due to a nonsynonymous mutation in SeMALRI that hampered the expression of SeMALT genes [69].

3. Mimic of Lager Yeasts by Artificial Hybridization

In addition to S. pastorianus, other hybrids have been isolated in brewing environments, such as hybrids between S. cerevisiae and Saccharomyces kudriavzevii from Belgian Trappist beers [70] or Saccharomyces bayanus (S. eubayanus × S. uvarum) hybrids isolated as contaminants from beer [38,71]. Taking all these natural hybrids as templates, novel synthetic interspecific hybrids have been constructed in laboratories to combine desired phenotypes in a single clone. Compared to parents, interspecies hybrids often show 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 of the ability of organisms to buffer the effects of external perturbations through metabolic, physiological, or developmental adjustments; phenotypic novelty, additivity, and mid-parent phenotypes (semidominance) for some traits [72]. The most common approaches to performing sexual hybridization are mass-mating, rare-mating, and spore-to-spore mating [73]. In general, the first step is the yeast sporulation to generate gametes; then the spores can merge in a zygote after being randomly shuffled (mass-mating) or after being physically placed in contact with each other (spore-to-spore mating) or even after a fortuitous homoygosis (rare-mating). The success of these techniques is strictly affected by reproductive isolation [74,75], so the parental strains are selected within the same genus in order to maximize the hybridization yield.

De novo \( S. \text{ cerevisiae} \times S. \text{ eubayanus} \) hybrids were successfully constructed for lager-brewing [45,76–81]. 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 a complex and enriched aromatic profile. However, most of de novo \( S. \text{ cerevisiae} \times S. \text{ eubayanus} \) hybrids also produced 4 VG, which confers smoky flavor to beer. This sensorial attribute, also called “phenolic off-flavor” (POF), is often negatively perceived in lager beer style. The majority of wild \( S. \text{ cerevisiae} \) strains and all known \( S. \text{ eubayanus} \) strains characterized so far exhibit a POF\(^+\) phenotype. Three strategies successfully overlooked this detrimental trait. Krogerus et al. [77] used rare-mating to obtain fertile allotetraploids, which produced allodiploid spores to backcross with the POF\(^-\) parent. This method is quite time-consuming as it requires complementary auxotrophic derivatives of parental strains and at least two breeding rounds. Alternatively, Diderich et al. [82] exploited UV mutagenesis to select POF\(^-\) \( S. \text{ eubayanus} \) mutants that were crossed with a POF\(^+\) \( S. \text{ cerevisiae} \) parental strain. Although the POF\(^-\) phenotype was selectable based on the low ability of mutants to grow in the presence of ferulic acid, this approach also requires time-expansive screening steps. 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]. Despite this cutting-edge approach, 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].

Hybrids alternative to \( S. \text{ cerevisiae} \times S. \text{ eubayanus} \) were also proposed to combine cold- and sugar-tolerance. Cold-tolerant \( \text{Saccharomyces} \) species including \( \text{Saccharomyces arboricola} \), \( \text{Saccharomyces mikatae} \), and \( \text{Saccharomyces uvarum} \) were used as surrogates of \( S. \text{ eubayanus} \) in crosses with \( S. \text{ cerevisiae} \) [85,86]. Sato et al. [85] performed mass-mating between top-fermenting \( S. \text{ cerevisiae} \) yeasts and a cryotolerant \( S. \text{ uvarum} \) strain and selected hybrid candidates by combining the \( S. \text{ uvarum} \) contribution for melibiose assimilation with the \( S. \text{ cerevisiae} \) contribution for growth ability at 35 °C. The resulting \( S. \text{ cerevisiae} \times S. \text{ uvarum} \) hybrids outperformed \( S. \text{ cerevisiae} \) 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 \( S. \text{ arboricola} \) and \( S. \text{ mikatae} \) as \( \text{Saccharomyces} \) non-\( S. \text{ cerevisiae} \) counterparts in hybridization cross. Although the rare-mating technique should give allotetraploid hybrids, hybrids with variable ploidies (from 2 to 4n) were obtained and those with higher ploidy levels overcame the 2n hybrids in fermentative vigor. Interestingly, \( S. \text{ arboricola} \)- and \( S. \text{ mikatae} \)-derived hybrids performed well in wort, although their cold-tolerant parent strains did not have any capabilities of utilizing maltose or maltotriose. All hybrids increased desirable aroma-active esters, but exhibited POF\(^+\) phenotype.

4. Evolutionary Engineering

Evolutionary engineering techniques have been extensively used to improve wine and sake yeasts and, recently, were also adopted in brewing to increase sugar utilization [87], flavor profile [88], and stress tolerance [89–92]. For instance, residual amounts of maltotriose are detrimental for breweries as it increases the probability of beer spoilage. Continuous cultivation of \( S. \text{ pastorianus} \) strain CBS...
1483 on a maltotriose-enriched sugar mixture enhanced maltotriose uptake and utilization in evolved derivatives, which, consequently, showed lower residual maltotriose and higher ethanol yield than the parental strain [87]. Similarly, Bleck et al. [89] 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 high ethanol concentrations and maltose and maltotriose as the sole fermentable sugars, two typical conditions of the final stages of very high-gravity fermentation. Selected yeast variants exhibited improved fermentation performance in a very high-gravity (24 °P) wort, avoiding sluggish fermentation at the end of the brewing process. Brewer’s yeast variants exhibiting faster and more complete brewer’s wort fermentative performance were also obtained by recursive cultivation of lager EMS-mutagenized yeast in the presence of high sorbitol amount [90]. More recently, genetic instability of de novo S. cerevisiae × S. eubayanus hybrids was exploited by cultivation under high ethanol concentration to gain high ethanol-tolerant derivatives for lager-style beer production [79].

The adaptive evolutionary approaches described above modify phenotypes that have a direct adaptive impact on yeast survival or growth. Other evolutionary engineering strategies are “directionless” and entail the usage of drugs and analog compounds, which are not directly related to the increase in the desired phenotype. Gibson et al. [88] exposed repeatedly a lager strain to a sub-lethal level of chlorsulfuron, in order to gain derivatives with reduced diacetyl production. Diacetyl resulted from spontaneous decarboxylation of α-acetolactate and is responsible for the unpleasurable buttery flavor in lager-style beer. Chlorsulfuron inhibits the acetohydroxy acid synthase Ilv2, which catalyzes the conversion of pyruvate to α-acetolactate. Tolerance to chlorsulfuron may result in either higher or lower diacetyl production as this phenotype is not expected to have a direct impact on the yeast survival or fermentation performance. Similar “directionless” approaches were used to improve flavor profile in sake yeasts (Table 1), but require the accurate and extensive screening of evolved strains before their industrial exploitation.

| 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-farnesylypyridinium          | 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 alternative food matrices, to produce beers with novel flavor profiles and other desirable properties [98–104]. Yeast isolation represents one of the most interesting solutions for brewers, since it takes advantage of the natural biodiversity of the microorganisms adapted to grow in their habitats. On the other hand, knowledge of molecular mechanisms underpinning some relevant beer-related traits in ale and lager yeasts has been highly improved in recent years. These genotype–phenotype correlation maps can assist the accurate and marker-assisted selection of natural variants with the highest aptitude for brewing at least partially avoiding time-consuming trial-and-error procedures (Figure 3).

Although S. cerevisiae yeasts from various alcoholic beverages, such as Cachaça spirits [98], wine [99–101], pulche, tequila, or sake [102], were proposed for brewing, 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 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]. Several S. cerevisiae sourdough strains were able to ferment glucose, maltose, and trehalose. Interestingly, the trehalose uptake is carried out by the same transporters as uptake maltose and maltotriose, 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 locus and in IMAI gene copies, which encodes a major isomaltase. These pieces of evidences suggested that bread and beer strains could share a 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 an S. cerevisiae yeast isolated from sourdough, which overcame the commercial brewing yeast in ethanol content, lowering the pH and production 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 of sourdough yeasts are that (i) baker’s yeasts do not exhibit the flocculation trait required for brewing [109]; (ii) they generally possess a POF + phenotype. These features make sourdough strains suitable for brewing beer specialties, such as wheat beers, lambic beers, and 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

A further trend in costumed-designed starter culture entails the usage of non-Saccharomyces yeasts, or non-conventional yeasts. These yeasts have been conventionally considered detrimental for fermented alcoholic beverages as they negatively impact sensorial properties, such as the turbidity, viscosity, or mouthfeel [110–114]. However, appropriate strain selection and accurate management of fermentative parameters can give novel products with alternative aromatic tastes that fulfill the modern consumer’s expectations to receive a product with enhanced aroma profile without chemical additives. Although non-conventional yeasts have been extensively used as bio-flavoring agents in wine-making [115–117], only in recent years some studies tried to apply them to brewing processes [113,118,119]. Compared to Saccharomyces, these yeasts generally show lower ethanol yield, so they are rather used in co-fermentation or in sequential fermentation with classical Saccharomyces brewing yeasts then as pure starter cultures. Otherwise, this low ethanol yield is not inconvenient to be overlooked, rather it can be exploited to produce to low-alcoholic (0.5%–1.2% v/v) or even alcohol-free (<0.5% v/v) beers, which are increasingly demanded beverages [120]. For instance, Saccharomyces ludwigii [121] and Pichia kluyveri [122] inefficiently fermented maltose and maltotriose and were successfully used to produce alcohol-free beers with rich flavor. Similarly, Zygosaccharomyces rouxii consumed ethanol under aerobic conditions and produced actively desired flavor compounds, leading to low-alcohol and flavorful beers [123].

The most investigated non-conventional yeasts for brewing purposes belong 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 species. 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 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 spoilage yeasts are responsible for the so-called “Brett flavor” in wine and soft drinks. “Brett flavor” is a 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 4 VG, 4-vinylphenol and 4-vinylcatechol), substituted tetrahydropyridines (including 2-ethyltetrahydropyridine, 2-acetyltetrahydropyridine, 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 in wort and also hydrolyze glucoside-bound monoterpenes, which are present in brewers’ wort that comes from hops [124]. The breakdown of these bonds releases monoterpenes, which became 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 yeasts have been recently 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, the co-culture of S. cerevisiae and T. delbrueckii in 1:20 ratio increased the production of ethyl decanoate and ethyl dodecanoate, leading to specialty beer with a flavor distinct from conventional ales (Table 2) [114]. Callejo and co-workers [128] reported that S. pombe overcame T. delbrueckii, L. thermotolerans, and S. ludwigii in alcohol content, as well as foam consistency and persistence. Domizio et al. [129] proposed the usage of L. thermotolerans pure culture 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 with 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 lager yeast WS34/70 single culture, improving the fruity flavor perception in the final product [130].

| 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, AWRII499 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-culture 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 kudriarzewii x12, X9035 pure/sequentially inoculated with Ale 514 brewing yeast [118] |
Table 2. Cont.

| Yeast                        | Strain                          | Fermentation Conditions                                           | Reference |
|------------------------------|---------------------------------|-------------------------------------------------------------------|-----------|
| *Saccharomyces ludwigi*      | DBVPG 3010, DBVPG 3304, DBVPG 3398, DBVPG 3931, DBVPG 4116, DBVPG 6721 | pure                                               | [120]     |
| *Starmerella bacillaris*     | X9029                            | pure                                                              | [118]     |
| *Starmerella bombicola*      | VI0.2Y A1                        | pure                                                              | [118]     |
| *Torulaspora delbrueckii*    | DiSVA 254                        | pure/co-culture 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-culture with *S. cerevisiae* starter strain US-05          | [119]     |
| *Zygosaccharomyces rouxii*   | DBVPG 4084, DBVPG 6187, DBVPG 6424, DBVPG 6463, DBVPG 6921 | pure                                                              | [120]     |
| *Zygotorulaspora florentina* | DiSVA 263                        | pure/co-culture with *S. cerevisiae* starter strain US-05          | [119]     |
| *Zygotorulaspora florentina* | X9022                            | pure/sequentially inoculated with Ale 514 brewing yeast            | [118]     |

7. Conclusions

This study provided an overview of the main non-genetic engineering techniques used so far to meet the challenging requests for brewing yeast diversification in the emerging craft beer market. Synthetic *S. cerevisiae × Saccharomyces* non-*cerevisiae* hybrids, non-conventional yeasts, and *S. cerevisiae* natural variants from alternative bioreservoirs represent the most promising frontiers for craft brewing, as they impact and significantly enrich the aroma profile of the final products. We also showed 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 in order to complement pro and contra of every single technique.

**Author Contributions:** Conceptualization, L.S.; writing—original draft preparation, F.I., M.C., and L.S.; writing—review and editing, L.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by a grant from AEB Spa, Brescia, Italy.

**Conflicts of Interest:** The authors declare no conflicts of interest. The funders had no role in the design of the study, in the writing of the manuscript, or in the decision to publish the review.

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