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YEAST EXTRACTS

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Old yeasts, young beer—The industrial relevance of yeast chronological life span

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Abstract

Much like other living organisms, yeast cells have a limited life span, in terms of both the maximal length of time a cell can stay alive (chronological life span) and the maximal number of cell divisions it can undergo (replicative life span). Over the past years, intensive research revealed that the life span of yeast depends on both the genetic background of the cells and environmental factors. Specifically, the presence of stress factors, reactive oxygen species, and the availability of nutrients profoundly impact life span, and signaling cascades involved in the response to these factors, including the target of rapamycin (TOR) and cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathways, play a central role. Interestingly, yeast life span also has direct implications for its use in industrial processes. In beer brewing, for example, the inoculation of finished beer with live yeast cells, a process called "bottle conditioning" helps improve the product's shelf life by clearing undesirable carbonyl compounds such as furfural and 2-methylpropanal that cause staling. However, this effect depends on the reductive metabolism of living cells and is thus inherently limited by the cells' chronological life span. Here, we review the mechanisms underlying chronological life span in yeast. We also discuss how this insight connects to industrial observations and ultimately opens new routes towards superior industrial yeasts that can help improve a product's shelf life and thus contribute to a more sustainable industry.

KEYWORDS
bottle conditioning, chronological life span, flavor stability, PKA pathway, TORC1/Sch9, yeast

1 | INTRODUCTION

When nutrients are abundant, *Saccharomyces cerevisiae* typically replicates through mitosis, by means of an asymmetric process known as budding (Piper, 2012). Asymmetric cell division leads to asymmetric inheritance of cellular components, with the newly formed cell receiving freshly synthesized cellular material. By contrast, the mother cell retains most of the old and damaged cellular components and gradually loses the capacity to form new buds. The number of budding events that a cell can undergo before irreversibly turning quiescent is defined as its replicative life span (RLS) (Kaeberlein, 2010; Mortimer & Johnston, 1959). The limited number of budding events that mother cells can undergo is generally attributed to the accumulating effect of oxidation, aggregation and degradation of proteins, the loss of vacuolar acidity and its associated decline in mitochondrial function, and the appearance of extrachromosomal ribosomal DNA circles (ERCs) (for a schematic overview, see Figure 1) (Hughes & Gottschling, 2012; Kaeberlein, 2010; Steinkraus et al., 2008). ERCs are formed by homologous recombination within the ribosomal DNA (rDNA), which results in a self-replicating circular DNA molecule (Kaeberlein, 2010; Steinkraus et al., 2008). The exact mechanism by which ERCs accelerate aging remains to be elucidated, but they are thought to interfere

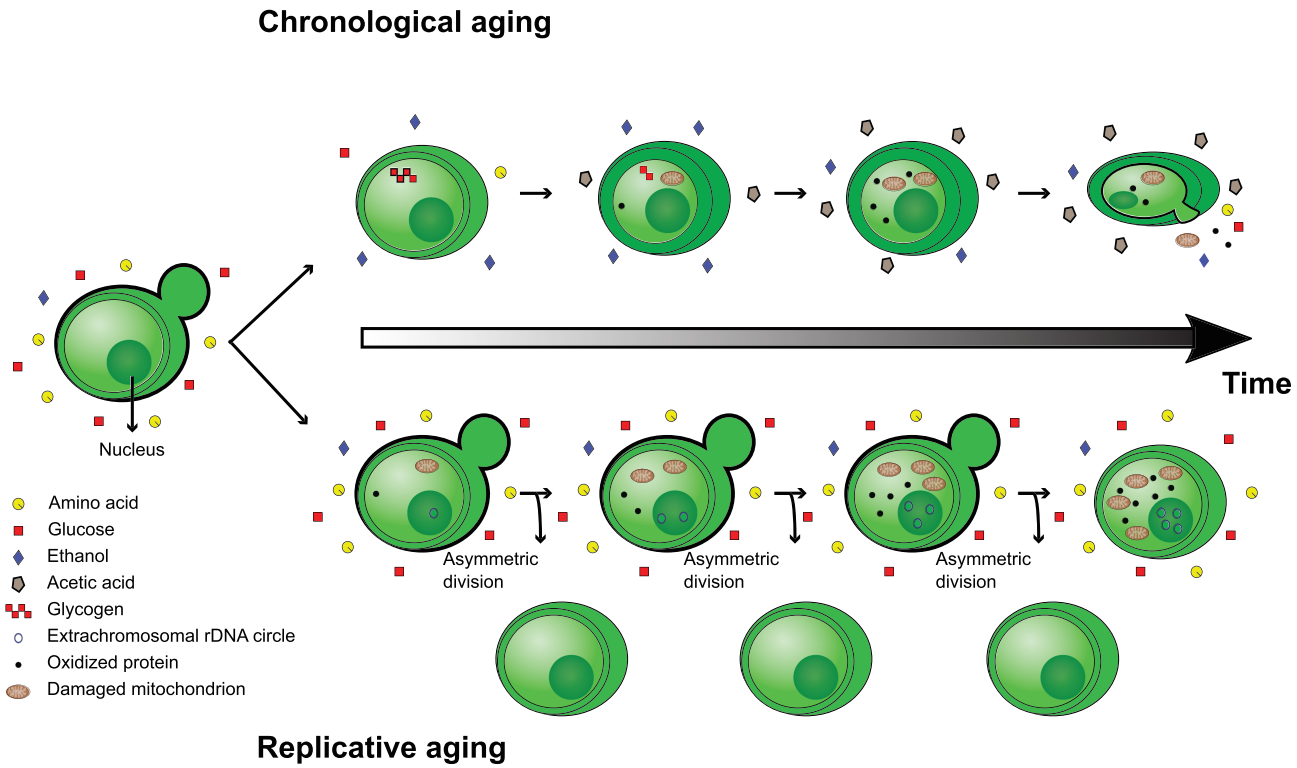

FIGURE 1 The two aging paradigms of *Saccharomyces cerevisiae*. Chronological aging and the chronological life span (CLS) refer to the time a yeast cell can remain viable in a nondividing state. This G_0-like state is usually induced by nutrient limitation or external stressors and is characterized by an increased resistance to multiple stressors. Over time, stored carbohydrates become depleted, and the external pH decreases due to the metabolism of ethanol to acetic acid. Combined with cellular damage (e.g., oxidized proteins and mitochondrial damage) that inevitably arises upon extended survival, these environmental factors ultimately lead to cell lysis. Replicative aging and replicative life span (RLS) refer to how many doublings a mother cell can undergo before senescence. This number is limited due to extrachromosomal ribosomal DNA circles, oxidized proteins, and damaged mitochondria that accumulate over time. Upon division, the damage is asymmetrically inherited by the mother cell, which allows the daughter cells to reset their replicative capacity, irrespective of the RLS of the mother cell

with regular rDNA by destabilizing it and competing for transcription factors (Kaeberlein, 2010). The number of asymmetric divisions a mother cell can undergo when cultivated on rich agar medium was estimated to be approximately 25 (Mortimer & Johnston, 1959; Zhang et al., 2012), although the precise number depends on the genetic background of the strain (Stumpferl et al., 2012).

In an industrial context, a second, alternative measure for yeast aging, namely, the chronological life span (CLS), may be more relevant than the RLS. In finished beer (as well as in many natural environments), yeast cells are not continuously dividing but instead spend much of their lifetime in a reversible quiescent induced by the limitation of essential nutrients (de Virgilio, 2012). The length of time a cell can remain viable in this state is known as its CLS (see Figure 1) (Fabrizio & Longo, 2003; Longo et al., 2012). Although the underlying mechanisms likely differ, both aging measures, RLS and CLS, reflect an innate limitation in the amount of damage a singular cell is able to tolerate before its cellular functions are irreversibly compromised. This raises the question as to what underlying mechanisms are involved and if these processes can be halted or reduced to increase life span and, ultimately, the industrial performance of yeast cells.

Yeast serves as a prime model for eukaryotic cell aging. Specifically, yeast RLS constitutes a model for the aging as it occurs in dividing cells like fibroblasts or lymphocytes, whereas CLS serves as a model for aging of postmitotic cells such as mature neurons and muscle cells (Kaeberlein, 2010; Longo et al., 2012; Ruetenik & Barrientos, 2015). As such, there is an extensive body of knowledge on the genetic factors affecting CLS. To date, close to 2000 genes have been related to CLS (Saccharomyces Genome Database, 2021), hinting at the broad range of cellular processes that are involved in aging, including mitochondrial function, amino acid homeostasis, phosphate sensing, glycogen accumulation, apoptosis, tRNA methylation, chromatin modification, purine biosynthesis, iron metabolism, cell cycle regulation, target of rapamycin (TOR) signaling, protein kinase A (PKA) signaling, and autophagy (Alvers et al., 2009; Campos et al., 2018; Fabrizio et al., 2010; Garay et al., 2014; Gresham et al., 2011; Jung et al., 2018; Matecic et al., 2010; Powers et al., 2006). Which of these cellular processes are relevant for CLS at a given time depends on the conditions to which the cells are subjected during aging (Smith et al., 2016).

To our knowledge, there have not yet been any studies aimed at characterizing the pathways governing yeast CLS in industrial context of beer conditioning, typically characterized by stresses such as nutrient scarcity, high ethanol concentrations, and variable temperatures (Dekoninck, 2012; Rogers et al., 2016). As a result, it is unknown

whether there are specific cellular processes that are crucial for surviving during bottle conditioning and subsequent storage or whether CLS is primarily governed by cellular pathways that have proven to be relevant in other media as well. In this review, we will first focus on the primary molecular mechanisms and environmental parameters known to influence yeast CLS that have been confirmed by multiple labs. In a second part, we connect these findings to a brewing setting and discuss the role that CLS could play in the flavor stability of beer.

2 | ENVIRONMENTAL FACTORS AND GENETIC PATHWAYS INFLUENCING CLS

CLS is measured when cells are in a nondividing state that is usually the result of starvation for an essential nutrient (carbon, nitrogen, sulfate, phosphate, biotin, or other spore elements) (Sun & Gresham, 2021). This causes cells to arrest within the G_1 phase of the cell cycle and transition to the stationary G_0 phase, characterized by a so-called core quiescence program as a means to cope with environmental stresses (de Virgilio, 2012; Gray et al., 2004). This "core quiescence program" includes the production of reserve carbohydrates, such as glycogen and trehalose, thickening of the cell wall, induction of autophagy, and several other changes that prepare the cells for extended survival (de Virgilio, 2012; Werner-Washburne et al., 1996). It has to be noted that a stationary phase culture is often heterogeneous, with only a subpopulation of cells being truly "quiescent" cells (Allen et al., 2006; Li et al., 2013; Sun & Gresham, 2021). These quiescent cells are typically small, unbudded daughter cells that arose from an asymmetric last division in response to glucose depletion (Li et al., 2013; Miles et al., 2021). They are longer lived than cells that did not properly induce quiescence and are more dense, for a large part due to a higher amount of reserve carbohydrates (Allen et al., 2006; Shi et al., 2010). Importantly, proper entry into a quiescent state is not achieved upon abrupt starvation but requires the cells to experience a gradual nutrient depletion. Given the large influence of the environment in general and nutrient specifically, it is not surprising that genes and pathways involved in the regulation of the cell cycle, nutrient signaling, and stress response play a major role in CLS. Two of the most important pathways involved in age regulation include the PKA pathway and the TOR complex 1 (TORC1)/Sch9 pathway (Deprez et al., 2018; Longo et al., 2012; Swinnen et al., 2014; Wei et al., 2008). Interestingly, analogous pathways of both are shown to also influence aging in more complex organisms, including worms, flies, and mice (Kenyon, 2001; Longo & Fabrizio, 2002; Sinclair, 2005).

2.1 | PKA pathway

The PKA pathway orchestrates gene expression in response to environmental cues (Conrad et al., 2014; Thevelein et al., 2008). In general, under favorable, nutrient-rich conditions, it stimulates properties associated with rapid fermentative growth and downregulates properties connected to slow respiratory growth or stationary phase (Conrad et al., 2014; de Virgilio, 2012; Thevelein et al., 2008). When nutrients become limited, for example, during the lagering or storage of beer, the PKA pathway would stimulate a reverse response.

The PKA pathway is influenced by all nutrients essential for growth, but is especially responsive to fermentable sugars, most notably glucose (Conrad et al., 2014). Both intracellular and extracellular glucose levels are signaled to Cyr1, an adenylate cyclase that catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP) when glucose is abundant. The messenger molecule cAMP can bind to the two regulatory subunits of the PKA complex (encoded by *BCY1*), thereby liberating the two catalytic subunits into the cytoplasm (encoded by either of three partially redundant genes *TPK1*, *TPK2*, and *TPK3*) (Conrad et al., 2014; Thevelein et al., 2008). In turn, these catalytic subunits directly phosphorylate cytosolic enzymes such as phosphofructokinase 2, pyruvate kinase, and fructose-1,6--bisphosphatase, through which PKA controls glucose metabolism (Conrad et al., 2014). Moreover, they also regulate gene expression at the transcriptional level through the (de)activation of several transcription factors. A crucial intermediate enzyme in this regard is the protein kinase Rim15. Once active, Rim15 induces typical G_0 traits such as cell cycle arrest and expression of stress resistance genes, primarily through control of the downstream transcription factors Msn2/4, Gis1, and Hsf1. Although Msn2/4 and Gis1 regulate genes containing a stress response element (STRE) or a postdiauxic shift (PDS) element in their promoter region respectively, Hsf1 enhances expression of genes with a heat shock element (HSE) within their promoter region (Conrad et al., 2014; de Virgilio, 2012; Pedruzzi et al., 2000; Thevelein et al., 2008; Wei et al., 2008). An overview of the PKA pathway is depicted in Figure 2.

An example of a PKA-mediated starvation response is the control of intracellular trehalose levels in response to glucose. When glucose becomes limited, reduced PKA signaling triggers Msn2/4-mediated transcriptional expression of the STRE-containing genes *TPS1* and *TPS2*, which encode trehalose-6-phosphate synthases (Rubio-Texeira et al., 2004). This stimulates the synthesis of trehalose, which has an important function in cellular stress resistance as it protects the membrane from desiccation, increases the thermal stability of proteins, and acts as an osmoprotectant (Hounsa et al., 1998; Verstrepen, Iserentant, et al., 2004; Wiemken, 1990).

2.2 | TORC1/Sch9 pathway

The second major nutrient signaling pathway involved in CLS is the TORC1/Sch9 pathway, shown in Figure 2 (Deprez et al., 2018; Fabrizio et al., 2010; Powers et al., 2006; Swinnen et al., 2014). TORC1 is composed of one of two kinase homologs, Tor1 or Tor2, and three regulatory subunits (Kog1, Lst8, and Tco89). Together, they form a rapamycin-sensitive kinase complex that is responsive to stresses, nutrient quantity, and nutrient quality (Deprez et al., 2018; Urban et al., 2007). Although TORC1 is best known for its sensitivity

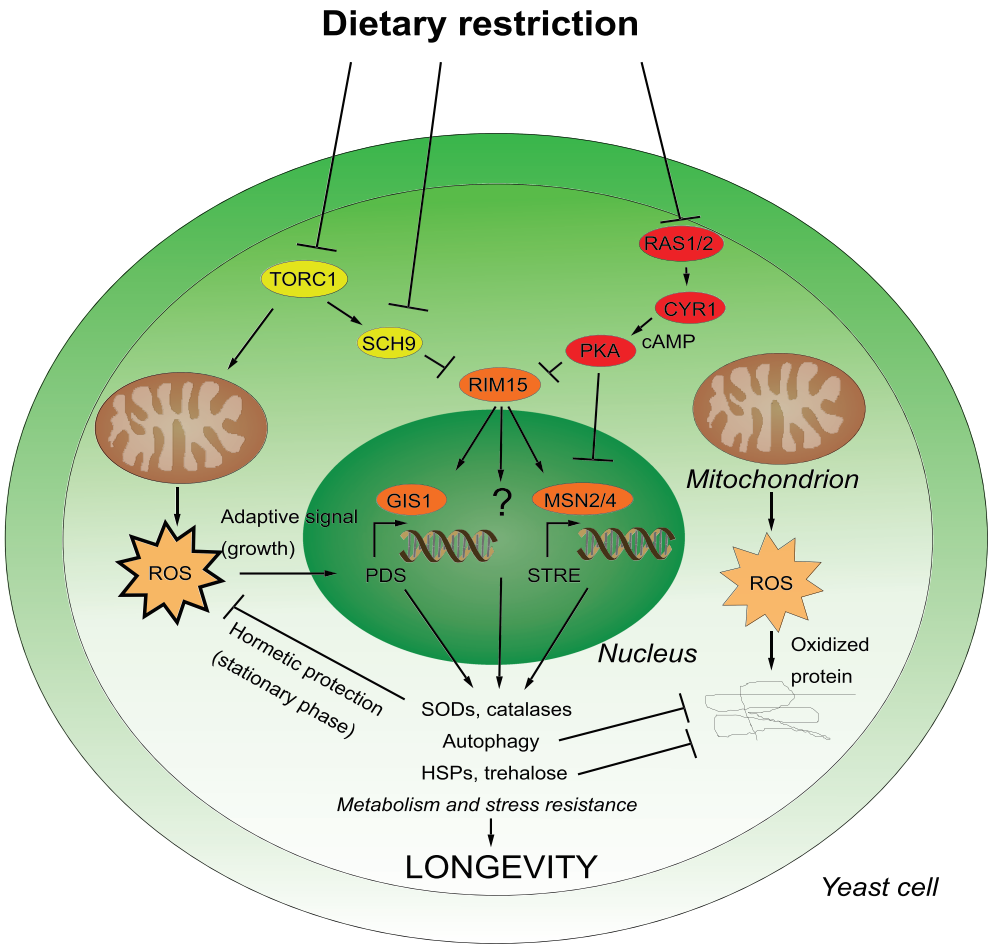

FIGURE 2 Major pathways driving chronological life span. Both the TORC1/Sch9 and PKA pathways are responsive to the availability of nutrients in the environment. Upon nutrient restriction, they are downregulated and allow the central protein kinase Rim15 to activate the stress responsive transcription factors Gis1 and Msn2/4. Together, these pathways orchestrate the cellular stress responses through increased transcription of, among others, heat shock proteins, trehalose synthase, autophagy-related genes, and detoxifying enzymes such as Sod2 and Ctt1. During growth, reduced TORC1/Sch9 signaling also upregulates mitochondrial respiration and consequently increases the formation of ROS. This stimulates an adaptive response that decreases ROS production during chronological aging. Together, these cellular changes result in an increased chronological life span

to amino acids, particularly glutamine and leucine, it is also responsive to other essential nutrients, including glucose (de Virgilio, 2012; Deprez et al., 2018; Loewith & Hall, 2011). How exactly amino acids are sensed and how signals are transduced to TORC1 remain poorly understood (Deprez et al., 2018; Prouteau & Loewith, 2019). The downstream effector branches of TORC1 on the other hand have been characterized in detail. The two main effectors are the Tap42-containing phosphatases and the kinase Sch9 (de Virgilio, 2012; Loewith & Hall, 2011). Together, they control several cellular processes such as stress resistance, autophagy, cell cycle progression, and amino acid metabolism in response to nutrient availability (de Virgilio, 2012; Loewith & Hall, 2011). For CLS, the Sch9 effector seems to be the prime regulator. Deletion of *SCH9* leads to a 300% increase in CLS compared with the wild-type strain, making it one of the most potent CLS-enhancing genetic interventions in *S. cerevisiae* (Fabrizio et al., 2001). Sch9 exerts its regulatory role for a large part through the protein kinase Rim15. As mentioned above, Rim15 integrates signals from both the TORC1/Sch9 and PKA pathways to control the cell cycle and orchestrate the cellular protection against environmental stresses via the transcription factors Msn2/4 and Gis1. Other physiological functions downstream of TORC1 that are implicated in CLS include pH homeostasis, stress resistance, cell size and cell cycle progression, sphingolipid metabolism, mitochondrial function, and autophagy (Conrad et al., 2014; Deprez et al., 2018; Garay et al., 2014; Gonzalez & Rallis, 2017; Huang et al., 2012; Loewith & Hall, 2011; Swinnen et al., 2014). The latter two are discussed in more detail below.

2.3 | The role of mitochondria in CLS

The primary role of mitochondria is to generate cellular energy in the form of ATP through oxidative phosphorylation. During oxidative phosphorylation, a set of enzymatic complexes transport electrons from high-energy molecules (such as NADH or $FADH_2$) to oxygen in a series of redox reactions, yielding water and cellular energy (Fernie et al., 2004). However, redox centers within this electron transport chain may leak electrons that can prematurely react with oxygen in nonenzymatic reactions, yielding superoxide anions (Turrens, 2003). Although $O^{-}_{2}{}^{\bullet}$ is not a highly reactive oxidant, it is a precursor of most other reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radicals, and is involved in the propagation of damaging oxidative chain reactions (Pan, 2011; Turrens, 2003). These oxidative reactions with ROS can irreversibly damage cellular components. Accumulation of such damaged cellular material, especially mitochondrial DNA, has been proposed as one of the primary mechanisms contributing to cellular aging (Bonawitz et al., 2007; Harman, 1956; Pan, 2011).

To alleviate the oxidative stress that inevitably arises as a consequence of respiration, yeast possess several enzymes, including the mitochondrial superoxide dismutase, Sod2, and the cytosolic catalase, Ctt1, to help detoxify ROS (Fabrizio et al., 2003). The genes coding for both enzymes contain an STRE in their promoter and are partially regulated by Msn2/4, linking them to the PKA and TORC1/Sch9 pathways.

Although ROS-induced cellular damage could offer an explanation for some studies observing a CLS decrease after aeration (Barré et al., 2020; Smith et al., 2016), the role of oxygen in aging is controversial and requires nuance. Indeed, if ROS were the main source of cellular senescence, one would expect that cells grow on a respiratory carbon source, which requires active (and thus ROS-producing) mitochondria to generate energy, would have a shorter CLS than cells that remain in a fermentative state for most of their life. However, growth on nonfermentable carbon sources has been shown to extend CLS (Pan, 2011; Piper et al., 2006; Smith et al., 2007). Moreover, respiratory-deficient mutants ("petites") were shown to be shorter lived than their respiration-competent parent strains (Bonawitz et al., 2007). One explanation for these observations is that nonfermentable carbon sources limit the amount of acetic acid that is formed during growth, which has been shown to have detrimental effects on prolonged yeast survival (Burtner et al., 2009; Sauer & Mattanovich, 2016). Another explanation for this seemingly counterintuitive phenomenon is the so-called hormesis effect, which states that a low dose of toxic stimulus induces resistance against ensuing stress of similar nature (Pan et al., 2011; Rattan, 2008). As such, a temporary and mild increase in superoxide generation during growth may elicit an adaptive CLS-extending response during stationary phase (Figure 2). This explanation is also consistent with the observation that fermentative growth in the presence of oxygen has been shown to extend CLS compared with growth under complete anaerobic conditions (Bisschops et al., 2015). For this reason, it could be hypothesized that freshly propagated yeast (i.e., yeast that has recently been subjected to relatively oxygen-rich conditions) is better prepared to cope with future oxidative damage than yeast that was harvested from fermentations tanks after the main fermentation. Exposure to oxygen may also help cells to accumulate sterols and unsaturated fatty acids that can contribute to overall fitness and CLS (Bisschops et al., 2015).

3 | INFLUENCE OF GENETIC BACKGROUND ON CLS

Most of the CLS-related genes and pathways discussed above were identified using lab strains or their derivatives. Although there are several powerful tools available that allow in-depth analyses of such strains, they are not representative of the true natural diversity in CLS between different *S. cerevisiae* strains (Schacherer et al., 2007). In a recent genome-wide association study (GWAS), nearly 1000 yeast isolates were probed for survival at three different time points, in three different conditions (De Chiara et al., 2020). Survival varied strongly between strains (0.5% to 96.8% survival after 20 days), a large part of which was explained by population stratification. For example, domesticated French dairy yeasts had fewer than 30% surviving cells after 7 days in part due to loss-of-function mutations in *SIC1*, a gene necessary for quiescence. French Guiana strains on the other hand still had more than 60% surviving cells after 21 days, likely because they harbored a defective *PCL7*, a gene that normally represses quiescence. The authors uncovered 292 such variants that control survival under quiescence, most of which were time and environment specific. The top GWAS hit was a highly diverged, nonreference allele of the cell wall glycoprotein Hpf1, which extended survival under calorie restriction at all time points (De Chiara et al., 2020).

Interestingly, together with Flo11, another cell wall glycoprotein, Hpf1 also stood out as the most important of 30 QTLs that governed the difference in CLS between a long-lived North American oak bark strain and a short-lived West African palm wine strain (Barré et al., 2020). The authors showed that massive intragenic tandem repeats in the HPF1 allele from the short-lived parent cause the cells to float during exponential growth as opposed to cells from the long-lived parent that remain sedentary. The buoyancy-induced increased oxygen exposure reduces the number of cells that properly enter a quiescent state and was shown to modulate lipid biosynthesis, purine biosynthesis, and methionine metabolism, thereby impinging on CLS (Barré et al., 2020).

In another QTL study, Jung et al. (2018) selected a Japanese sake strain and a White tecc tree strain from Ethiopia, which exhibited contrasting CLS phenotypes in media containing different concentrations of different carbon sources. Depending on the concentration and the type of carbon source, different QTLs were identified, confirming the existence of nutrient-dependent life span control mechanisms. For example, the sake strain was shortest lived when glucose was low (0.5%) but longest lived when glucose was higher (2% or 10%) or when galactose (2%) was used as a carbon source. The authors demonstrated that the strain harbors a nonsense mutation in *RIM15*, which prevented it from gaining the expected life span extension when glucose was low. However, a missense mutation in *SER1* permitted it to survive the longest within the media containing galactose or higher concentrations of glucose as a carbon source. Ser1 has a function in the serine biosynthesis pathway. The mutation resulting in the increased CLS can likely be attributed to metabolic reprogramming due to a blockage in the main serine biosynthesis pathway, which prevents extracellular acetate from accumulating and favors the buildup of high intracellular trehalose stores (Jung et al., 2018). Importantly, neither *FLO11* and *HPF1* nor *SER1* had been linked to CLS before, revealing the strength of using multiple approaches to identify genes linked to a specific phenotype.

4 | THE EXPERIMENTAL TOOLBOX TO ASSESS CLS IN YEASTS

There are several experimental designs, assays, and methodologies available to study the CLS of yeast. For a typical CLS assay, cells are

grown in SC medium with 2% glucose as the carbon source (Fabrizio et al., 2001; Garay et al., 2014; Longo et al., 2012). Because dietary restriction has been identified as a central factor linked to cellular aging, several experiments use glucose concentrations as low as 0.5% (Longo et al., 2012; Wei et al., 2008). Once the glucose is fermented to ethanol, the cells undergo a PDS and slowly start to metabolize the accumulated ethanol (de Virgilio, 2012). At the end of this period, most cells exit the G_1 phase of the cell cycle and prepare for an extended period of starvation, often complemented by extracellular stress arising from the accumulation of fermentation byproducts (e.g., high ethanol levels or low pH due to production of organic acids) (Longo et al., 2012; Murakami et al., 2011; Werner-Washburne et al., 1996). This is often regarded as the starting point for CLS assays. From that point on, the aging culture is sampled at different time intervals to monitor cell survival. Because CLS is strongly affected by the composition of the medium in which the cells are aged, its composition is often varied to gain insight into medium-specific CLS changes. Examples include aging cells in water, nutrient-limited laboratory media, or industrially relevant medium such as finished beer (Burtner et al., 2009; Campos et al., 2018; Jung et al., 2018; Mirisola et al., 2014).

There are different ways to monitor cellular survival. One such method, the colony-forming unit (CFU) assay, is often considered to be the gold standard. It involves plating an aliquot of the aging culture on solid medium and counting the colonies that form from the viable cells. This process is repeated until the CFU drops below a predefined value, usually between 0% and 10% of the original count (Mirisola et al., 2014; Piper, 2012).

Another commonly used method involves selectively staining the dead or viable cells in a culture and comparing them to the total number of cells. Certain dyes, such as 5-carboxyfluorescein diacetate (CFDA) or methylene blue, penetrate the membrane and rely on the metabolic activity of the cell to distinguish dead from viable cells. CFDA can be cleaved by intracellular esterases, thereby releasing carboxyfluorescein that stains viable cells fluorescently green (Breeuwer et al., 1995). Methylene blue, on the other hand, is a colorimetric stain sensitive to the redox environment. Viable cells can reduce the dye to its colorless counterpart, whereas dead cells are unable to do so and remain blue following exposure (Bapat et al., 2006; Kwolek-Mirek & Zadrag-Tecza, 2014). Although methylene blue is the industry standard, staining cells with the structurally similar methylene violet has been reported to result in less variation in color intensity, eliminating potential operator subjectivity (Smart et al., 1999; Van Zandycke et al., 2003). This method is regularly employed by brewers, who use a hemocytometer and microscope to estimate the viability of their industrial yeast batches prior to pitching. Other dyes, such as trypan blue or propidium iodide, rely on the integrity of the cell membrane to distinguish between dead and viable cells. Trypan blue is excluded from cells with intact membranes, whereas it enters cells with disrupted membranes and stains the cytoplasm blue (Kucsera et al., 2000). Similarly, propidium iodide selectively enters cells with a damaged membrane and intercalates between the bases of nucleic acids, thereby staining the cells fluorescently red (Davey & Hexley, 2011; Kwolek-Mirek & Zadrag-Tecza, 2014). Fluorescent dyes have the advantage over colorimetric dyes in that they allow for high-throughput measurements of CLS using a flow cytometer.

Finally, it has also been observed that the optical density obtained after incubating an aged yeast culture in fresh medium for a fixed period of time correlates with the number of viable cells present in the original aging culture (Powers et al., 2006). This concept of yeast outgrowth was further optimized and validated by Murakami et al. (2008) and Murakami and Kaeberlein (2009). Rather than using OD values, the authors calculated viabilities of the aging culture based on a shift in the outgrowth curve compared with the previous measured time point. Next to fluorescent dyes, these OD measurements also allow for relatively easy high-throughput measurements of CLS of different strains under different experimental conditions.

5 | RELEVANCE OF CLS FOR THE FERMENTATION INDUSTRY

Apart from being a eukaryotic model organism, *S. cerevisiae* is also at the core of many industrial applications such as brewing, baking, winemaking, and bioethanol production. In these processes, CLS plays a prime role because premature cell death can lead to diminished production efficiency and quality (Allen et al., 2010; Orozco et al., 2012). In the following section, we focus on the effect of yeast CLS in the brewing industry, with special emphasis on flavor stability.

5.1 | The flavor (in)stability of beer

Perhaps the most critical quality challenge that brewers face is achieving flavor stability (Bamforth & Lentini, 2009; Bamforth & Parson, 1985; Lehnhardt et al., 2018; Vanderhaegen et al., 2006). In contrast to wines, the aroma of beer starts to deteriorate almost immediately after production. The exact flavor evolution is beer specific and depends strongly on production, packaging, and storage parameters (Bamforth & Lentini, 2009; Bamforth & Parson, 1985). Nevertheless, general trends include a decline in bitterness, fruity and floral notes, and an increase in off-flavors like cardboard, ribes, honey, caramel, sherry, and bread (Bamforth & Lentini, 2009; Barnette & Shellhammer, 2019; Dalgliesh, 1977; Ferreira & Collin, 2020; Vanderhaegen et al., 2006). Most of these changes are attributed to increasing levels of flavor-negative carbonyl compounds in the beer, such as (E)-2-nonenal, acetaldehyde, and 2-methylbutanal (Hashimoto & Eshima, 1979; Vanderhaegen et al., 2006). They either can be formed de novo during storage, for example, through Maillard reactions or oxidation of higher alcohols, or can be the consequence of chemical degradation reactions of complex molecules that cause the release of smaller volatile compounds (Baert et al., 2015; Bamforth & Lentini, 2009; Suda et al., 2007). For example, many reactions that yield carbonyl compounds are favored by high temperatures and thus primarily occur during wort production. However, rather

than being stripped during boiling, they can bind to the amino group of an amino acid, peptide, or protein, yielding a nonvolatile imine that can persist in the final beer (Baert et al., 2015; Haefliger & Jeckelmann, 2013). Similarly, carbonyl compounds can form adducts with sulfites formed by yeast during fermentation. Gradual release of these carbonyl compounds in the beer during storage is also an important contributor to the development of aged flavor (Baert et al., 2012; Baert et al., 2015; Dufour et al., 1999).

Arguably, the most potent strategy to minimize beer aging is cooled transport and storage (Bamforth & Lentini, 2009). In a globalized market however, cold storage quickly becomes costly on top of not being very environment friendly. Other strategies to increase flavor stability aim to minimize the oxygen intake during brewing and bottling and to limit the thermal load on the wort during boiling (De Schutter, 2008; Saison et al., 2011; Wietstock et al., 2016). Although such strategies can significantly benefit a beers shelf life, they require specialized equipment and still leave room for further improvements. Interestingly, it has been observed that the addition of living yeast cells can decrease the aged flavor of already stale beer, whereas the addition of fresh yeast prior to bottling (i.e., bottle conditioning) can decelerate the subsequent aging process (Saison et al., 2011, 2010). This offers an interesting opportunity for beers that undergo secondary fermentation in the bottle, because the addition of yeast prior to packaging is part of the manufacturing process.

5.2 | Effect of bottle conditioning on beer aging

The mechanism through which *S. cerevisiae* is thought to affect flavor stability is threefold. First, it can act as oxygen scavenger, thereby reducing the oxidative reactions (e.g., via ROS) that take place during storage (Ahrens et al., 2018a, 2018b; Dekoninck et al., 2013; Marconi et al., 2016). Second, some strains can produce high levels of sulfite, which is a very potent flavor stabilizer due to both its antioxidant activity and its ability to convert carbonyl compounds into flavorless sulfite–carbonyl complexes (Baert et al., 2015; Guido, 2016; Kaneda et al., 1996; Saison et al., 2011). Whether the amount of sulfite formed during refermentation is sufficient to make a significant contribution to flavor stability has not yet been investigated. Lastly and arguably most importantly, *S. cerevisiae* possesses several enzymes with the ability to reduce flavor-negative aldehydes and ketones to their corresponding, less flavor-active alcohols (Saison et al., 2010; Vanderhaegen et al., 2003). The mechanism primarily involves aldoketoreductases (e.g., encoded by *ADH1*, *ADH6*, *ADH7*, *ALD4*, *GRE2*, *GRE3*, and *ARI1*) that, in the reduction process, reconvert the excess of reduced NADH and NADPH molecules, formed throughout refermentation, to their oxidized form (Chang et al., 2007; Liu, 2018; Saison et al., 2010; Van Iersel et al., 2000). This mechanism of coenzyme regeneration is also referred to as "yeast-reducing power." An overview of the mechanisms through which *S. cerevisiae* can act on flavor stability of beer is shown in Figure 3.

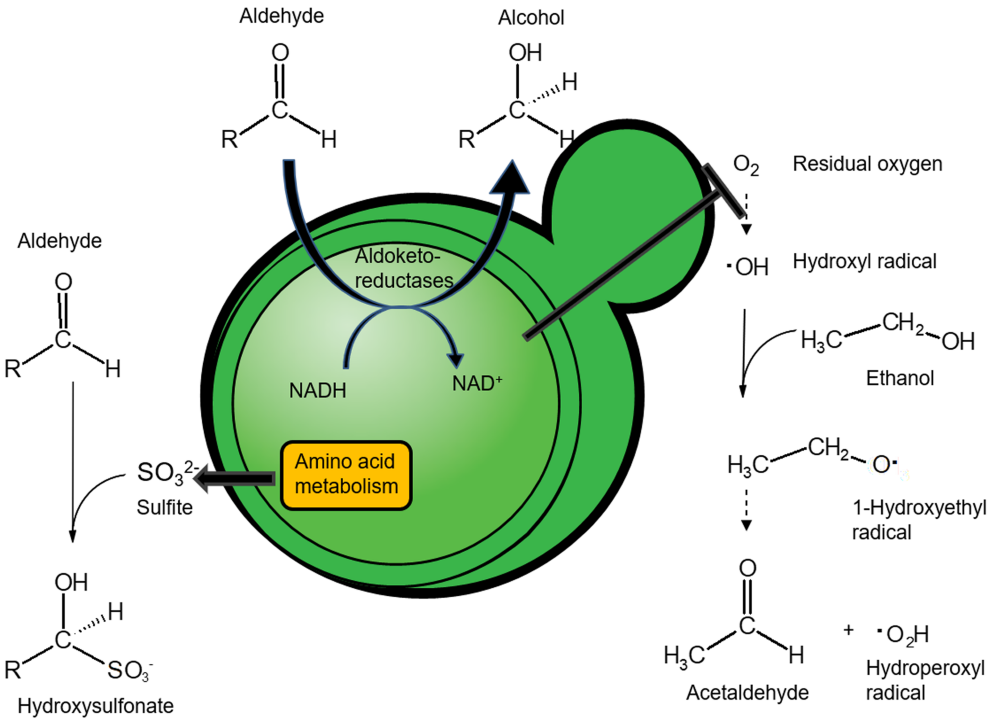

FIGURE 3 Overview of the mechanisms through which *Saccharomyces cerevisiae* can slow down beer aging. Left: Sulfite is formed as an intermediate during cysteine and methionine synthesis. Next to its role as an antioxidant, sulfite can form adducts with staling aldehydes, thus preventing them from staling the beer. Top: Coenzyme regeneration allows aldoketoreductase enzymes to convert flavor-negative aldehydes to their corresponding, less flavor-active alcohols. Right: By scavenging residual oxygen, *S. cerevisiae* can prevent oxidative reactions from both degrading beneficial aroma compounds and formation of undesired carbonyl compounds. An example of the ROS-mediated oxidation of ethanol (quantitatively the most important alcohol) to acetaldehyde is also shown

The reductive activity of yeast is very important during beer lagering, where it is responsible for reduction of the buttery diketone diacetyl to its corresponding flavorless alcohol, 2,3-butanediol (De Keukeleire, 2000; Krogerus & Gibson, 2013). However, Saison et al. (2010) showed that addition of yeast to aged beer also resulted in reduction of aldehyde levels (e.g., furfural, 2-methylpropanal, and methional) to levels similar to those present in the fresh beer. More importantly, they showed that aging beer in the presence of yeast (both with and without addition of sugar extract for the refermentation process) resulted in more than a twofold reduction in several staling aldehydes including 2-methylpropanal, phenylacetaldehyde, furfural, and 2-methylbutanal (Saison et al., 2011).

One major condition for yeast to exert its positive effect on flavor stability is, of course, that it remains viable, even after refermentation is complete and all fermentable sugars have been converted into alcohol and carbon dioxide, which typically occurs over 2 weeks (Dekoninck, 2012; Vanbeneden et al., 2006). This is where CLS becomes important. Depletion of the added refermentation extract also implies the start of a starvation period for the yeast during which the cells enter a nondividing stationary phase. Apart from nutrient scarcity, the cells must cope with low pH, high ethanol concentrations, high carbon dioxide pressure, and in some cases even extreme (high or low) storage temperatures, making extended survival very challenging (Dekoninck et al., 2013; Rogers et al., 2016). Current data concerning the viability of yeast after it is bottled are limited and difficult to compare given the use of different yeast strains, beers, storage conditions, and methods to analyze viability. For example, Dekoninck (2012) reported viabilities below 4% for eight tested strains after a 90-day storage period of a blond beer (9.9% alcohol by volume [ABV]) at 25°C. Vanbeneden et al. (2006), on the other hand, reported viabilities ranging from 14% to 77% after refermentation and subsequent storage of three blond beers (ABV ranging from 6.6% to 6.8%) for 6 months at 22–24°C.

Maintaining viable yeast cells throughout storage could not only delay the onset of aged flavor development from the formation/release of carbonyl compounds but could also protect the beer from negative quality attributes linked to yeast autolysis. These include the release of flavor-negative volatile fatty acids, hydrolases and proteases that might influence beer foam stability, and esterases that have been shown to negatively impact the ester profile during storage (Marconi et al., 2016; Neven et al., 1997; Van Landschoot et al., 2007).

5.3 | Increasing CLS of beer yeasts

When it comes to increasing the CLS of *S. cerevisiae* in a brewery setting, literature is scarce. Very often, brewers use the same yeast for the primary fermentation and refermentation (Dekoninck, 2012). This allows harvesting yeast from the fermentation vessel and avoids the need for a specific propagation of the refermentation yeast. However, because the conditions encountered during refermentation are very different from those of the primary fermentation (e.g., CO_2 pressure, ethanol content, and nutrient scarcity), it is very likely that some specific yeast strains may be better suited for refermentation than the yeast used for the main fermentation. In laboratory settings, for example, it was shown that CLS strongly depends on genetic background; out of a set of 734 strains, De Chiara et al. (2020) observed viabilities ranging from 0.5% to 96.8% after 20 days. Therefore, a solution for brewers may be to select a strain that is better able to withstand the harsh conditions encountered during refermentation.

However, a long CLS is not the only phenotype relevant for brewers. The choice of yeast strain also depends heavily on other characteristics, including flocculation and aroma formation. It is well documented that different yeast strains show different flocculation behaviors (Smukalla et al., 2008; Verstrepen & Klis, 2006; Verstrepen, Reynolds, & Fink, 2004). Ideally, yeast should flocculate strongly at the end of fermentation but less so during bottle conditioning as this reduces the contact with the beer and also results in unsavory flocs of yeast in the consumer's glass. Similarly, the formation of specific aroma compounds like esters, higher alcohols, organic acids, sulfur compounds, and vicinal diketones is also highly strain dependent (Canonico et al., 2014; Dzialo et al., 2017; Pires et al., 2014). Even though the effect of the refermentation yeast on the flavor profile of the beer is considerably smaller than the effect of the yeast used for primary fermentation (Marconi et al., 2016; Van Landschoot et al., 2004), brewers are not keen to change yeast strain out of fear of losing the "signature flavor" their beer is known for.

As is clear from the previous sections, CLS is not only genotype specific but also strongly dependent on environmental conditions. Thus, apart from selecting a specific, suitable yeast strain, another strategy could be to increase CLS of the cells by altering their environment. One way would be to change the sugar composition of the extract that is added for the secondary fermentation in the bottle. It has been shown that using syrups with high glucose content resulted in a higher viability during refermentation than using syrups that primarily contain fructose, sucrose and maltose (Van Landschoot et al., 2007). It has to be noted that this observation is somewhat counterintuitive and needs to be confirmed, as glucose normally limits CLS through strong induction of the PKA pathway (Conrad et al., 2014; Verstrepen, Iserentant, et al., 2004).

Because finished beer is an extremely harsh environment characterized by nutrient scarcity, high ethanol concentration, high carbon dioxide pressure, low pH, and sometimes high incubation temperatures, a better strategy than changing the sugar extract could be to preadapt the yeast for the stresses encountered in the bottle (Dekoninck, 2012; Rogers et al., 2016). A key concept in linking different stresses with yeast survival is the hormesis hypothesis, which states that a low dose of toxic stimulus induces resistance against ensuing stress of similar nature (Pan, 2011; Rattan, 2008). An important feature of the hormesis hypothesis is that the genes involved in the response to a specific stress are pleiotropic and thus can enhance resistance to multiple other stressors (López-Lluch & Navas, 2016; Rattan, 2008). For example, cells subjected to dietary restriction acquire heat and osmotic stress resistance (Smith et al., 2007).

This characteristic suggests that *S. cerevisiae* can be adapted to the challenging conditions encountered in the bottle by propagating under different, mildly stressful conditions before inoculating it for refermentation (Dekoninck, 2012; Rogers et al., 2016).

Several studies have demonstrated a major impact of the medium composition during growth on the subsequent survival of yeast cells during stationary phase. For example, the half-life of yeasts aged with γ-butyric acid as the sole nitrogen source was almost 5× longer than the half-life of yeasts aged with the rich nitrogen source ammonium, without major effects on growth kinetics (Campos et al., 2018). Furthermore, oxygenating the growth medium, subjecting yeast to a dietary restriction or changing from a fermentative to a respiratory carbon source, all have been found to result in beneficial effects on CLS (Piper, 2012; Smith et al., 2007; Wei et al., 2008). It is important to point out, however, that the cells in these studies are not transferred to a new growth medium—as is often the case for refermentation—but were instead kept in the same aging medium when their CLS was assessed. Whether the positive effect is maintained when cells are transferred to another medium where they resume growth has only been studied sporadically. It has however been shown that the use of freshly propagated yeast is preferred over both harvested and dried yeast (Van Landschoot et al., 2004). As touched upon above, this could be the case because freshly propagated yeast has in most cases recently been subjected to relatively oxygen-rich conditions. This could increase its stress resistance and thereby better protect it from taking oxidative damage (Pan, 2011). Moreover, propagation in a sucrose medium poor in nitrogen resulted in a longer lasting viability during refermentation than propagation in wort (Van Landschoot et al., 2004). Likewise, cells subjected to a mild ethanol stress during propagation showed an increased refermentation performance compared with the same strain propagated in the absence of ethanol. This might be linked to an increased amount of unsaturated fatty acids, ergosterol, and trehalose (Dekoninck, 2012). However, this was demonstrated using yeast extract peptone dextrose (YPD) as the propagation medium, whereas brewers typically use wort. Taken together, although several previous studies have revealed several interesting phenomena regarding the parameters affecting yeast aging, further research is needed to translate these findings to brewery settings.

6 | CONCLUSION

CLS is an extremely complex phenotype, regulated by a myriad of genes and environmental cues. *S. cerevisiae* has served as a model to investigate CLS in higher organisms and as such has proven very valuable to elucidate the underlying mechanisms, such as the involvement of the TORC1/Sch9 and the PKA nutrient-sensing pathways. When nutrients become limited or when other external stresses compromise cellular functions, these pathways cause cells to switch to a nondividing stationary state and activate defense mechanisms that prolong the cell's life span. In addition, the conditions during growth also influence subsequent survival. The best documented example is dietary restriction. Cells grown under low levels of one or more essential nutrients generally survive much longer than cells grown under rich conditions. From a practical point of view, this effect of cell pretreatment might be extrapolated to industry. Subjecting cells to a weak (nutrient) stress during growth to induce a long-lasting stress response could allow brewers to preadapt yeast for stressful applications such as refermentation. This way, yeast life span could be increased and, given the ability of yeast to scavenge leftover oxygen and reduce aldehydes to their corresponding alcohols, flavor stability could be improved. Still, the most important and easy gains may be found in the selection of specific bottle-conditioning yeasts that show a long life span in beer, and may therefore help preserve beer over longer periods of time, even in suboptimal storage conditions.

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CONFLICT OF INTERESTS

The authors are presently engaged in research aimed at generating yeasts with a superior chronological life span for industrial use, where the laboratories and project are in part financially funded by Duvel Moortgat, which may eventually lead to the development of products that may be patent protected and licensed.

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