

Review

Yeast Life Span and its Impact on Food Fermentations

Agustín Aranda ^{1,*}, Helena Orozco ^{1,2}, Cecilia Picazo ^{1,3} and Emilia Matallana ¹

^{1.} Institute for Integrative Systems Biology, I²SysBio. University of Valencia-CSIC, 46980 Paterna, Spain; helena.orozco@uv.es (H.O.); cecilia.picazo@iata.csic.es (C.P.); emilia.matallana@uv.es (E.M.)

^{2.} IATA-CSIC, 46980 Paterna, Spain.

^{3.} Department of Chemistry and Molecular Biology. University of Gothenburg, SE405 Gothenburg, Sweden

* Correspondence: agustin.aranda@csic.es

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Abstract: Yeasts are very important microorganisms for food production. The high fermentative capacity, mainly of the species of the genus *Saccharomyces*, is a key factor for their biotechnological use, particularly to produce alcoholic beverages. As viability and vitality are essential to ensure their correct performance in industry, this review addresses the main aspects related to the cellular aging of these fungi as their senescence impacts their proper functioning. Laboratory strains of *S. cerevisiae* have proven a very successful model for elucidating the molecular mechanisms that control life span. Those mechanisms are shared by all eukaryotic cells. *S. cerevisiae* has two models of aging, replicative and chronological. Replicative life span is measured by the number of daughter cells a mother can produce. This kind of aging is relevant when the yeast biomass is reused, as in the case of beer fermentations. Chronological life span is measured by the time cells are viable in the stationary phase, and this is relevant for batch fermentations when cells are most of the time in a non-dividing state, such as wine fermentations. The molecular causes and pathways regulating both types of aging are explained in this review.

Keywords: yeasts; *Saccharomyces cerevisiae*; aging; life span; wine; beer

1. Introduction

Aging and death are parts of the life cycle of all organisms. The performance of yeast used in food production is dependent on the degree of cell viability and vitality at each point of their biotechnological use, so life span is an important aspect to be considered. The first part of this paper describes aging types, their molecular causes, and the mechanisms regulating them [1]. The next part highlights the particular aspects that impact the aging of *Saccharomyces* during wine, beer and sake production. Finally, it mentions some aspects of nonconventional yeasts present in particular fermentations. Dough fermentation by baker's yeast to produce bread happens in a solid matrix and vastly differs from the typical liquid fermentations of alcoholic beverages. Very little information is available about the cellular longevity issue under these conditions, but as stress conditions and mechanisms share common elements [2], the main factors explained herein should be in place in other fermentation types, such as that which happens in bread making. The same may apply to other industrial fermentations conducted by *S. cerevisiae*, such as bioethanol production.

2. Aging in *Saccharomyces cerevisiae*

As it is a relevant model organism, a great deal of work has been done in the molecular causes and regulation of aging in *Saccharomyces cerevisiae* [1,3,4]. *S. cerevisiae* is known as a budding yeast for its asymmetric cellular division type. Mother cells are able to produce a limited number of daughter

cells (around 20–25) by giving way to a mitotic aging model that is not related to time, but to the number of replications a cell can endure [1]. This is called the replicative life span (RLS). The other aging model is related to time and defined as the length of time a nondividing cell can maintain viability and reenter proliferation when conditions it allow to do so. This is called the chronological life span (CLS) [5]. Both models are related as they are controlled by the same pathways (see below), and chronological aging over a long period in the stationary phase leads to a reduction in RLS [6]. The standard method for RLS measurement involves the repeated isolation of the mother cell by micromanipulation [1,7]. The number of bud scars can be used to measure replicative aging but also to isolate mutations involved in lifespan [8]. CLS is usually measured by assessing the cellular viability by colony counts after growth in rich media [1,7], although high-throughput methods based on optical density measurements have been used to screen large collections of mutants [9].

The damage that causes senescence, and eventually death, may be of different origins and can vary among both aging paradigms. Damage is retained in mother cells during the RLS, and allows the rejuvenation of daughter cells, while it increasingly accumulates in the stationary phase with time [4]. It has been proposed that damaged mitochondria and oxidized proteins are molecular causes in both the CLS and RLS. The Free Radical Theory of Aging supports the classical view that oxidative damage caused by reactive oxygen species (ROS) is the ultimate molecular cause of aging and ulterior death [10]. ROS can damage all biological molecules, from proteins to membrane lipids, and that damage can be irreversible when DNA is damaged. This theory has been made obsolete by more recent theories and the discovery of additional molecular mechanisms (see below), but the relevance of oxidative damage is still an issue in the life span research field. Oxidatively damaged proteins are selectively retained in mother cells during cytokinesis [11]. Aggregation of misfolded proteins is attached to mitochondria and, during mitosis, well-anchored mitochondria help to retain these misfolded proteins, while the mitochondria entering daughter cells are free of aggregates [12]. Genomic stability has been linked to aging, and ribosomal DNA stability seems a particularly key factor during the RLS. Homologous recombination in rDNA can cause the so-called extrachromosomal rDNA circles (ERCs) to form, which are asymmetrically retained by mother cells [13]. Metabolism seems to play a key role in the molecular causes of the CLS. Ethanol has been proved to be an important player in aging [14], while the acidification caused by acetic acid has been proposed as the determinant cause of aging [15]. However, the key role of acetic acid has been dismissed by other studies and it remains an unsolved issue in the field (see reference [1] for a complete discussion on this issue). Glycerol, however, plays a beneficial role in the CLS [16]. Its production lowers metabolite flow from glucose fermentation into ethanol and acetic acid, causing also the increase of the concentration of NAD⁺ (a sirtuin activator, see below) [17].

The only intervention that has proven useful to extend life span from yeast (both the RLS and CLS) to primates to date is dietary restriction (DR); i.e., reducing a particular or the total nutrient intake without causing malnutrition [1,18]. When the number of calories is lowered, this intervention is referred to as calorie restriction. This fact suggests the considerable relevance of metabolism in aging, as well as the key role on life span of nutrient-sensing and signaling pathways, as explained in the next section.

3. The Genes, Pathways and Molecules that Regulate Life Span

As aging is a multifactorial process, the molecular mechanisms that regulate it are many, different and intertwined in a complex manner. As much as 2% of nonessential genes extend the RLS when deleted (like hexokinase) which merely reinforces this fact [19]. The key players in both the RLS and CLS are nutrient-sensing/signaling pathways as they are at the center of the events triggered by dietary restriction and are the targets of some of the most promising anti-aging drugs [1,3]. The most prominent of these pathways are RAS/cAMP/PKA and Tor/Sch9. The activation of these pathways promotes cell division and growth by stimulating protein synthesis and activating metabolism [20]. Glucose triggers cAMP production by adenylate cyclase, which is regulated by GTPases like Ras, and cAMP stimulates Protein Kinase A. Similarly, the Target of Rapamycin (TOR) kinase forms a complex called the TORC1 complex, which is activated by the presence of amino acids to regulate a variety of

cellular events through several kinases, particularly S6 kinase Sch9 that regulates translation, among other processes. The mutations of the key components of these pathways cause life span to extend, even when nutrients are abundant, which indicates that they are the regulators of dietary restriction [21,22]. The key targets downstream of these pathways are protein synthesis, stress response, mitochondrial function and proteostasis. Reduced mRNA translation is a cause of extended life span in both aging models [1]. The deletion of the *SCH9* gene or the inhibition of TORC1 with the drug rapamycin extends the RLS and causes the amount of polysomes to diminish [23]. Both the main nutrient signaling pathways repress the stress response by down-regulating Rim15 kinase, which controls general stress response transcription factors Msn2/4 [20,24]. The role of oxidative stress and the impact of the reactive oxygen species (ROS) produced by mitochondria in aging are complex and controversial. The overexpression of antioxidant enzymes like superoxide dismutase can extend the CLS [25]. However, the production of mitochondrial ROS in the growth phase seems to act as signaling molecules to promote CLS extension in an aTORC1 controlled manner [26]. Calorie restriction increases respiration and ROS production [27], thus the presence of reactive species per se is no hallmark of aging [26]. However, some proteins involved in redox control are relevant to DR. Calorie restriction induces H₂O₂ tolerance, but fails to efficiently extend the life span in cells lacking peroxidase Tsa1 [28]. Tsa1 is the main cytosolic peroxiredoxin, an enzyme able to degrade hydrogen peroxide that forms part of the thioredoxin system for redox control. The control of proteostasis, the correct degradation of damaged proteins or the recycling of proteins into amino acids during starvation, is relevant for aging. An increase in proteasome activity extends the RLS [29], while autophagy is required to achieve a full CLS under certain conditions [30].

Besides nutrient pathways, the most relevant group of aging genes includes NAD-dependent protein deacetylases called sirtuins. They are named after the SIR2 gene in *S. cerevisiae*, whose overexpression was first shown to extend the RLS [31]. Their main targets are histones, and they promote chromatin condensation by suppressing the formation of ERCs and enhancing genomic stability [32]. However, there are other targets of acetylation outside the chromatin that may play a role in aging. For instance, Sir2 is necessary for oxidatively damaged proteins in mother cells [11,33], but the situation in the CLS may differ as *SIR2* deletion extends the life span under calorie restriction conditions, and its deletion causes ethanol in the medium to be rapidly depleted, which may help to promote the CLS [14].

The fact that some natural molecules can modulate the activity of proteins related to the regulation of aging opens up an interesting research field. TOR kinases are defined by their repression by the macrolide rapamycin, a well-known antifungal and immunosuppressant molecule. It is widely accepted that rapamycin is able to extend the CLS in *S. cerevisiae* due to its inhibition of the TORC1 complex [9]. Inhibition of amino acid biosynthetic proteins, like the inhibition of glutamine synthetase by methionine sulfoximine, also inhibits some TORC1 functions [34], and is able to extend yeast's life span [9]. Another inhibitor of TOR, metformin, may also be potentially used as a life span modulator [35]. The most popular and controversial of these nutritional life span improvers is resveratrol. This is a plant polyphenol that has been proposed as a caloric restriction mimetic that may act against age-associated diseases [36]. It was thought that resveratrol activated sirtuins to promote life span extension [37], but this finding has been strongly disputed [4]. Lithocholic bile acid (LCA) has been identified as a molecule that prevents aging in yeast [38]. This lipid is not produced by *S. cerevisiae*, but is incorporated into mitochondrial membranes to protect cells. Therefore, lipids may also play an important role in aging.

4. Chronological Life Span in Wine Yeasts

Grape juice fermentation to produce wine is a typical growth type in batch, where yeasts displace the bacteria and filamentous fungi present on grape berries to transform sugars into ethanol [39]. At first, many non-*Saccharomyces* species are present, but finally *Saccharomyces*, and especially *S. cerevisiae*, impose themselves given their high fermentative power and good tolerance to stress [40]. The strains used for winemaking differ genetically from the ones used in the laboratory in several features, as they are prototrophic, homothallic and mostly diploid (although some aneuploidies have

been detected in commercial strains). *S. cerevisiae* shows several growth phases during wine fermentation: after a short lag phase, an exponential phase of cell proliferation, followed by a stationary phase with no growth that eventually leads to cell death [41] (Figure 1). The current modern winemaking trend is to inoculate grape juice with a selected starter of good fermenting yeasts in the form of active dry yeast (ADY). This makes the growth phase very short as cells divide around 4–6 generations. For this reason, the RLS is no limiting factor here. There is not analysis on the RLS of the yeast that impose on spontaneous fermentations, and it may be interesting to study this aspect. RLS may also be a parameter of interest during biomass propagation to produce ADY, but no study has yet been conducted on this topic. The bulk of sugar fermentation takes place after cells enter the nondividing state due to nitrogen limitation and/or ethanol accumulation. The viability and vitality of yeast in the stationary phase are relevant factors to help achieve full fermentation. So, the yeast CLS is an interesting issue during winemaking. Cell death is a not a very well understood phenomenon. Some authors have described how cells start dying when sugars are still present [41], while others claim that the death phase happens only when fermentation is over [42]. This controversy is probably based on the intrinsic genetic variability of different commercial wine yeast strains regarding the CLS [43]. When different strains are tested in the standard synthetic complete (SC) medium used for CLS experiments, a wide variety of death profiles are found. Long-lived strains are more tolerant to oxidative stress, while short-lived ones display increased adenylate cyclase activity. Hence their PKA is up-regulated [43]. On average, the yeasts isolated from industry and nature tend to have a shorter CLS than the most widely used laboratory strains, and the average RLS of natural isolates is about 30% longer than for laboratory strains [44].

Many environmental factors influence life span during winemaking [45] (Figure 1). Low pH and heat shorten the maximum wine yeast CLS, while hyperosmotic shock extends it. As grape juice is acidic with high osmotic pressure due to the abundance of sugars, both factors can compensate one another, at least partially in life span terms. Ethanol or acetic acid shortens the wine yeast CLS, as has been described for laboratory strains (see above), but acetaldehyde also does this [45]. Surprisingly, grape polyphenols quercetin and resveratrol negatively impact life span, even though they are well-known antioxidants [45].

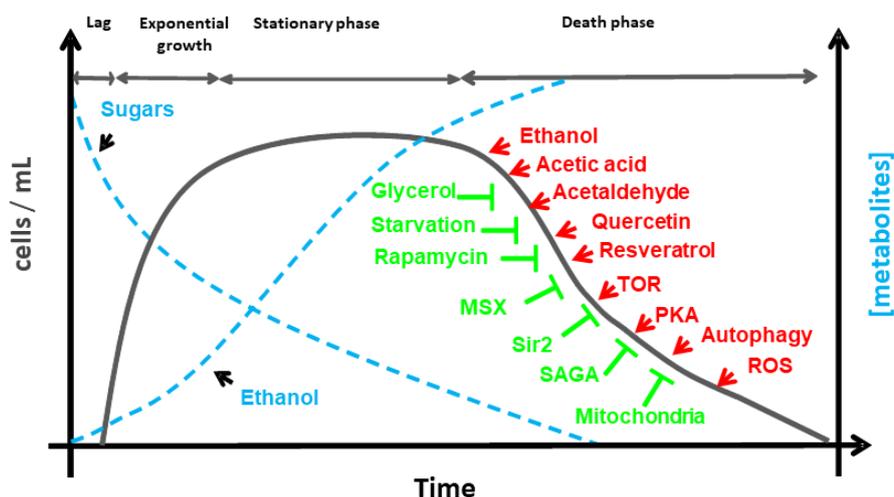


Figure 1. Growth phases of wine yeast during grape juice fermentation, changes in key metabolites and main aging regulators. Curves of cell viability (grey line), sugar consumption and ethanol production (blue dotted lines) during the fermentation times are shown. Positive regulators of the chronological life span are indicated in green and pro-aging factors are indicated in red.

Nutrient-signaling pathways strongly impact the wine yeast CLS. Besides, the aforementioned relevance of PKA [43], TORC1, and its regulated processes are particularly interesting. Nitrogen reduction triggers the CLS under winemaking conditions, which indicates

that dietary restriction is also in place in biotechnological environments [46]. TORC1 is one of the main signaling pathways to respond to nitrogen abundance. Its partial repression with methionine sulfoximine (see above) also prolongs the CLS, which indicates the role of this complex during life span regulation in wine yeasts. Likewise, herbicide glufosinate ammonium, which also targets glutamine synthetase, is able to extend the CLS in wine yeast [47]. The deletion of *SCH9* is known to cause a long CLS under laboratory conditions [21], and the same is true when this gene is mutated in wine yeasts [48]. However, the *SCH9* deletion mutant has a shortened CLS during grape juice fermentation. When nitrogen reduces by 25-fold in standard SC laboratory medium to mimic the C/N ratio in grape juice, then the CLS also shortens [48]. Therefore, the function of TOR/Sch9 pathways in aging depends on the balance of nutrients. For instance, nitrogen excess enhances yeast cell death in lipid-limited must, and *SCH9* deletion increases viability for this condition [49]. A set of micronutrients, like oleic acid, ergosterol, pantothenic acid and nicotinic acid, in limiting amounts are responsible for cell death when nitrogen is high, and TOR inhibition once again prevents this outcome [50]. Other players may be linked to metabolism and aging. The deletion of cytosolic thioredoxin causes the down-regulation of glycolysis during fermentation, which leads to life span extension [51]. The function of mitochondria is also required to achieve a full life span during winemaking, and this extension once again depends on Sch9 [48]. The impact of the mitochondrial genome on oxidative stress tolerance and life span can also be tested in hybrids with mitochondria from different species, like *S. cerevisiae* and *S. uvarum*. [52]. The hybrids with *S. cerevisiae* mitochondrial inheritance display increased tolerance to oxidative stress and extended chronological longevity.

Other factors beyond nutrient-sensing pathways that affect life span under wine fermentation conditions, and may at least partially channel the positive effects of dietary restriction. One of them is the enzymes devoted to acetylation and deacetylation, mainly histones. Sirtuins also play a role in life span under winemaking conditions, but not always the expected one. *SIR2* deletion extends the maximum chronological life span in wine yeasts grown on laboratory media, but shortens it during grape juice fermentation [46]. Its homolog, deacetylase *HST2*, has the opposite effect when deleted. The *SIR2* gene overexpression is able to extend the CLS under winemaking conditions while reducing the amount of acetic acid, suggesting that acetic acid is relevant for aging during grape juice fermentation [53]. The deletion of one of the main acetyltransferases, *GCN5*, extends the life span under winemaking conditions [46]. This enzyme is the catalytic subunit of a bigger complex involved in gene transcription regulation, SAGA, whose integrity is required extended longevity [48].

Autophagy is a key factor for longevity on laboratory growth media. When mutations that prevent autophagy are tested in those conditions, CLS is shortened [30]. However, the opposite effect happens under winemaking conditions. When this process is blocked through the mutation of one of the involved genes (*ATG7*), longevity is extended [46]. Therefore, the right amount of autophagy is required, and too much of it can be harmful under some environmental conditions. The underlying discrepancy between these results may be caused by the different ratio between nutrients in grape juice, poor in nitrogen and rich in sugars, the opposite than laboratory media. Apoptotic factors like the yeast metacaspase Yca1 play an unexpected positive role in wine fermentation as its deletion shortens the CLS during grape juice fermentation [53]. All these discrepancies can be related to the different nutrient balances present in grape juice, as explained above for *SCH9* deletion. Pub1 is an mRNA binding protein involved in stress granule formation, whose deletion increases glycerol production and extends the CLS during winemaking [54].

Cell aging and death are particularly relevant when producing sparkling wines prepared by the *champanoise* method, which involves a second fermentation inside the bottle after adding sucrose. In this environment, cell lysis contributes to the final wine organoleptic properties [55,56]. Nutrient starvation causes the activation of autophagy in this fermentation kind, and eventually leads to cell death and lysis [57]. The overexpression of autophagy-related genes, such *ATG3* and *ATG4*, has proven a useful way to increase cell lysis for nutrient starvation under sparkling winemaking conditions [58]. Cell death in this environment is also regulated by nutrient-sensing pathways as the

mutations in PKA regulatory subunit *BCY1* (a repressor of kinase activity) induce autolysis under simulated second-fermentation conditions [59].

5. Replicative Life Span in Brewing Yeasts

Like wine fermentation, beer fermentation is a batch fermentation by *Saccharomyces* (*S. cerevisiae* in many cases, and *S. pastorianus* for lager production) on a natural substrate, an aqueous extract of malted barley, plus hops, called wort [60]. As wort is poorer in sugars than grape juice, carbon and other nutrients do not abound at the end of fermentation, the yeast enters a quiescent state, which is much more similar to a canonical stationary phase under laboratory conditions. Therefore, the mechanisms described for the CLS may take place. At the end of fermentation, cells are cold-stored (3–4°C) in beer before being used again. The yeast which has been stored as a highly concentrated cell suspension is referred to by brewers as aged. If yeast biomass handling is not properly done, it may lead to yeast autolysis [61]. This is not a beneficial trait, as considered in sparkling wine production, because only 5% of autolysis negatively impacts beer organoleptic properties [61]. A transcriptomic analysis during autolysis indicates a decrease in the genes devoted to energy production, protein synthesis and stress response, while cell wall biogenesis, starvation response and DNA damage response genes are activated [61]. A proteomic analysis done in the same circumstances shows a similar pattern, which suggests that these changes are transcriptionally regulated [62]. Among the proteins up-regulated during autolysis, it is interesting to indicate the presence of peroxiredoxin Tsa1 as the only stress response protein present.

After storage, the biomass is reused, or repitched as it is called, and cells resume growth. So, the replicative aging of cells and the effect of the CLS on the RLS are relevant issues. This serial repitching is usually done between seven and 20 times, so aging issues of the reused biomass may be of much importance [63,64]. Different industrially used brewer's yeasts show a phenotypical variety in terms of both chronological and replicative life spans [65] as a maximum RLS may vary from 28 to 55 generations. Therefore, the selection of yeast starters according to the genetic differences that underlie these phenotypic variations may be useful to extend repitching practices. No correlation between the RLS and CLS was found in brewer's yeasts, which happens in laboratory strains, and chronological aged cells presented a shortened RLS [65].

In addition to strain selection, the way the biomass is collected offers a chance to modulate the age of the yeasts used in the next inoculation. Wort fermentation is carried out in fermenters known as cylindro-conical vessels (CCV) given their conically-shaped bottom. As older cells tend to be bigger and aggregate more, they go to the bottom of the CVV first, together with debris, dead yeasts and broken cells [64]. This has been proven right from counting bud scars by flow cytometry [66]. This bottom fraction is discarded and older cells are differentially removed. However, decantation tends to lose smaller virgin cells, particularly if it is done before cooling down the vessel (warm cropping). Hence the time and way that cropping is performed would alter the average replicative biomass age. Older cells tend to have an extended lag time before fermentation is resumed than younger cells due to an extended cell cycle, which causes breweries economic loss [64]. However, experiments done with synchronized cells have indicated that virgin cells are related with delayed sugar utilization compared to older cells, but the final gravity of the fermentation is similar. In contrast, virgin cells show lesser flocculation capacity [64]. Recent studies claim that serial repitching may not always be so detrimental. After 135 generations, a lager strain showed no significant differences from fresh cells in fermentative capacity terms and its genome did not alter, which indicates that some strains are more tolerant to genetic drift than others [67]. A large-scale serial repitching analysis done in three different breweries suggested that, on average, cells did not progressively age during extended serial repitching because the cells with large numbers of scars did not increase, and the ratio of virgin cells remained around 50% [68]. This would indicate that rejuvenation processes take place, such as autophagy, telomere silencing, and telomere protection genes. These may depend on different brewing practices like wort aeration, which is the best abiotic factor to explain rejuvenation [68]. Transcriptomic changes took place with the up-regulation of the genes involved in glycogen and trehalose synthesis after six runs [68].

Aeration may help rejuvenation, but it comes with a price. Oxidative stress is relevant in the first fermentation stages due to exposure to oxygen. Tolerance to oxidative insult is necessary to avoid respiratory incompetent petite cells from appearing when cells are exposed to oxygen [69]. The petite ratio rises during chronological aging, but only when oxygen is present. However, the ROS level decreases during autolysis, which means that oxidative damage does not cause this process [62].

6. Molecular Regulation of the Stationary Phase during Sake Fermentation

Sake is a rice-based alcoholic drink produced by the fungus *Aspergillus oryzae* which degrades starch, and by the yeast *Saccharomyces cerevisiae* which ferments the resulting monosaccharides. A transcriptomic analysis of yeast during sake production led to the identification of a defective general stress transcription factor *MSN4* gene in industrial sake strains [70]. This allelic variation favored the enhanced ethanol fermentation rate of these strains. However, the downside of this increased fermentative power is deficiency when cells enter the stationary phase after growth ceases, compared to laboratory strains [71]. This defect is caused by a truncated version of protein kinase Rim15p in sake strains [72]. This kinase controls those changes that lead to an entry in the quiescent state, including ethanol fermentation cessation. Its overexpression with a gluconeogenic promoter has been used to enhance the fermentative capacity in yeasts with different uses [73]. Rim15 modification in this direction is also able to increase brewer's yeast performance [74]. Natural variations in this gene between wine and sake strains may explain the physiological traits noted between these two kinds of yeast, which may affect aging, like nitrogen utilization or glycerol production [75].

7. Aging in other Yeasts

Beyond the industrial strains of *Saccharomyces* devoted to fermented foods, very little is known about aging on other yeast species used to produce more specific foods. However, increasing interest is being shown in other yeasts as aging model systems [76], some of which may contribute to the production of other food types. For instance, the fission yeast *Schizosaccharomyces pombe* is interesting for the RLS given its symmetrical cell division. *S. pombe* may be present at the beginning of wine and other spontaneous fermentations of traditional foods, and is valued for its ability to, for instance, reduce malic acid. A dairy producing yeast, *Kluyveromyces lactis*, is used as a model for the CLS and it is a Crabtree negative yeast, and offers an alternative vision about the influence of nutrients on aging. For instance, the reduction of sugars does not extend the chronological longevity in this yeast, unlike what happens in *S. cerevisiae* [77]. Other dairy fermenting yeasts, such as *Kluyveromyces marxianus*, show typical markers of oxidative stress (increased ROS accumulation, high glutathione and superoxide dismutase) when reaching the stationary phase in whey medium [78], which suggests similar chronological aging to that described for *S. cerevisiae*. Therefore, common themes may influence the aging of each yeast under industrial conditions, but the specific contribution of each yeast's genetics and the different biotechnological environments have to be taken into account.

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