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Review

Genetic, Physiological, and Industrial Aspects of the Fructophilic Non-*Saccharomyces* Yeast Species, *Starmerella bacillaris*

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Abstract: *Starmerella bacillaris* (synonym *Candida zemplinina*) is a non-*Saccharomyces* yeast species, frequently found in enological ecosystems. Peculiar aspects of the genetics and metabolism of this yeast species, as well as potential industrial applications of isolated indigenous *S. bacillaris* strains worldwide, have recently been explored. In this review, we summarize relevant observations from studies conducted on standard laboratory and indigenous isolated *S. bacillaris* strains.

Keywords: grape must; fermentation; wine; yeast; non-*Saccharomyces*; *Starmerella bacillaris*; *Candida zemplinina*

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1. Introduction

Starmerella bacillaris (syn., *Candida zemplinina*; [1]) is a non-*Saccharomyces* yeast species frequently found in grapes and fermenting grape musts [2–6]. *S. bacillaris*, mainly isolated from sweet botrytized wines [3,7–10], has also been isolated from low-sugar fermenting grape musts from *Vitis vinifera* and other *Vitis* species [4,11–13], as well as from fruits, fruit-associated insects, and soil [3,14–17].

S. bacillaris was isolated and identified in Napa Valley (California, USA) from botrytis-affected wine fermentations (strain EJ1 [18]) and, shortly after, from fermenting botrytized grape musts in Zemplin, Hungary [9]. Following detailed morphological, physiological, and molecular characterization, the Hungarian isolates, which share D1/D2 sequences (i.e., large rDNA subunit) [19] with only two nucleotides of difference with the EJ1 Californian isolate, were proposed as part of the novel species, *Candida zemplinina* [9]. Additional studies (i.e., isoenzyme profiles, 26S rDNA restriction profiles and 26S rDNA sequencing), performed on the type strain of *C. zemplinina* (i.e., CBS 9494) and on two cultures of the neotype of *Saccharomyces bacillaris* (i.e., CBS 843 and PYCC 3044), confirmed that these three strains correspond to the same species. Because *Saccharomyces bacillaris* is the older epithet and has nomenclatural priority over *C. zemplinina*, Duarte et al., (2012) reinstated *Sacch. bacillaris* as *Starmerella bacillaris* comb. nov., with *C. zemplinina* as an obligate synonym [1].

Further studies have widely recognized *S. bacillaris* species (syn., *C. zemplinina*, type strain 10-372 ^{T} = CBS 9494^{T} = NCAIM Y016667^{T}) as a major non-*Saccharomyces* yeast at initial stages of spontaneously fermenting grape musts [6,11,20,21]. *S. bacillaris* has also been identified, along with *Saccharomyces cerevisiae*, at the end of alcoholic fermentations [22], suggesting that, in mixed fermentations with *S. cerevisiae*, *S. bacillaris* may coexist longer than other non-*Saccharomyces* species [22].

S. bacillaris has been described as an acidogenic, fructophilic, psychrotolerant, and highly osmotolerant microorganism [9,23,24]. The great genetic biodiversity found in isolates from different environments and the vast physiological diversity encountered in metabolic characterizations have contributed to interest in using this species as an industrial co-starter in fermented beverage production [7,8,25,26]. *S. bacillaris* has been characterized

as a safe microorganism, which may encourage to its use as a biocontrol agent of several food pathogens [27].

In this review article, we discuss recent results from research into the genetics and genomics, ecology, population genetics and geographical biodiversity, metabolism, and ethanol production, as well as the biocontrol potential of *S. bacillaris*.

2. Genetic Aspects

2.1. Starmerella/Wickerhamiella Clade

S. bacillaris has been recognized as part of the W/S (*Wickerhamiella* and *Starmerella*) clade that branches close to *Yarrowia lipolytica* in the *Saccharomycotina* species tree [28]. A detailed phylogenetic tree of the W/S clade has recently been published [28]. Members of this clade are characterized by limited nutritional versatility, an unusually small cell size, and a strong association with the floral niche (i.e., flowers and insects that visit flowers) [28]. The *Starmerella* genera forms a well-supported subclade, with species usually presenting fermentative capacity, a trait normally absent in *Wickerhamiella* [28]. According to a comprehensive phylogenetic tree based on ITS1 sequences of 3942 fungal species, this *Starmerella* subclade does not include other enological non-*Saccharomyces* yeast species, suggesting a strong genetic divergence from the large wine yeast group [29]. The specific features present in *Starmerella* versus *Wickerhamiella* subclades probably result from the remodeling of important fluxes in central carbon metabolism as well as the reinstatement of other metabolic pathways [28]. Thus, the genera *Starmerella* harbors an unusually large number of genes of alien origin, which were shown to reconstruct the fermentative pathway in *S. bacillaris* [30], as well as other metabolic pathways, as shown in *Starmerella bombicola* [31,32]. For example, most of the genes in the thiamine salvage pathway in distinct subclades within the W/S lineage were originally acquired from bacteria by either horizontal gene transfer (HGT) or horizontal operon transfer (HOT) events [32]. In fact, *S. bacillaris* species lacks both *THI5* and *THI4* genes, required for de novo thiamine synthesis, but harbors the salvage pathway bacterial genes, *THI6* and *THI20*, which allow the assimilation of thiamine derivatives from the environment [32]. Other potential HGT events in *S. bacillaris* populations, evolving in alternative non-conventional enological (i.e., non-*V. vinifera*; [11]), floral, and high sugar ecosystems, remain to be explored.

2.2. S. bacillaris Genome

S. bacillaris has three chromosomes [33]. A mean genome size of 9.3 Mb and G+C content of 39.4% have been recognized following sequencing of the genomes of *C. zemplinina* type strain CBS 9494 [2] and strains FRI751, PAS13, PYCC 3044, and NP2 [30,34–36]. Electrophoretic karyotyping showed the presence of two chromosomes of ~4 Mb each and one chromosome of ~1.8 Mb [33]. Genome alignment of two *S. bacillaris* strains (i.e., FRI751 and PAS13) evidenced the presence of five main chromosomal translocations, close to genes involved in mitochondrial and nucleotide metabolisms (i.e., endoplasmic reticulum protein-retaining receptor (KDEL), biosynthesis of aromatic amino acid protein (Aro1p), farnesyl diphosphate synthase (FDPS) protein, dUTP pyrophosphatase, cytochrome c oxidase subunit 2, and mitochondrial ribosomal protein of the large subunit) [29]. The chromosome alignments analyzed showed a general synteny between both genomes, with a high number of single nucleotide polymorphisms (SNPs) (i.e., 33,771 variants among 1146 putative genes) [29]. These observations highlight the large potential biodiversity among wild isolates of *S. bacillaris*, as has been reported by other authors [37–39].

2.3. S. bacillaris Ploidy and Microsatellite Loci

S. bacillaris has shown no evidence of sporulation ability [9], and it is considered haploid [38]. Interestingly, allele characterization for microsatellite loci CZ15 and CZ59 in *S. bacillaris* strain 11-6 suggested apparent heterozygosis for both loci [38]. A recent detailed analysis of *S. bacillaris* microsatellite loci CZ15 and CZ59, however, highlighted the molecular basis for the observed apparent heterozygosity [39]. The study, which in-

cluded the analysis of the 10 *S. bacillaris* reference polymorphic microsatellite loci (i.e., CZ1, CZ4, CZ11, CZ13, CZ15, CZ20, CZ33, CZ45, CZ54, and CZ59; [38]), revealed a higher degree of structural complexity than expected from previous descriptions of yeast microsatellite loci [40–44]. In fact, alleles of alternative *S. bacillaris* microsatellite loci contain, in addition to the expected, and/or sometimes absent, variable lengths at their internal tandem-repeated motifs (TRM), extensive variations consisting of additional SNPs and/or insertions/deletions (*indels*), largely contributing to allelic variations [39]. In the case of loci CZ11 and CZ59, these alternative non-TRM sequences may explain the observed apparent heterozygosity in certain strains [39]. Interestingly, extension of these studies to microsatellite loci of *S. cerevisiae* and other common enological non-*Saccharomyces* species (i.e., *Brettanomyces bruxellensis*, *Hanseniaspora uvarum*, *Meyerozyma guilliermondii*, *Saccharomyces uvarum*, and *Torulaspora delbrueckii*) showed the existence of similar sequence and structural variants, potentially contributing to allele diversity [39]. These studies indicated that allele sizing of TRM polymorphic yeast microsatellite loci using PCR, although valid for strain differentiation and population genetic studies, does not necessarily score the number of units at their TRM [39]. Moreover, sequence analysis of microsatellite loci alleles could be used in evolutionary and phylogeny studies of yeast species [39].

2.4. S. bacillaris Species and Strain Identification

In addition to microsatellite loci, a large repertory of molecular strategies has been used to identify *S. bacillaris* isolates worldwide (Figure 1). Among these, restriction fragment length polymorphism (RFLP) analyses of 5.8S-ITS regions (Table 1) enable the identification of *S. bacillaris* isolates to species level, as well as differentiation of this yeast species from the close species, *Candida stellata* (i.e., *Dra*I and *Mbo*I enzymes) [33] and *Starmerella bombicola* [45] (Table 1). Other molecular strategies have been used to characterize *S. bacillaris* to strain level, including SAU-PCR, RAPD-PCR, micro/minisatellites, and Rep-PCR, as well as AFLP-fingerprinting, mt-DNA-RFLP, and TRtRNA-PCR analyses [8,16,17,23,37,38,46]. Fingerprinting analyses, using SAU-PCR and Rep-PCR, have enabled recognition of genetic similarity between isolates from different sources [17,23] (Figure 1). Similarly, RAPD-PCR and SAU-PCR analyses showed a relative genetic homogeneity within Italian strains, with no differences in terms of strain clustering or geographic distribution [8]. The combination of different molecular strategies (i.e., polymorphic minisatellite loci, RAPD-PCR fingerprinting, and microsatellite primer $(GTG)_5$ analyses) had a marked impact in population genetics analyses in *S. bacillaris* [16,46] (Figure 1). In addition, in situ fluorescence hybridization (FISH), targeting rRNA, has been optimized and validated as a culture-independent technique to monitor and identify *S. bacillaris* in biological samples [47].

2.5. S. bacillaris Ecology

A detailed intraspecific genotype analysis in a large number of indigenous isolates of *S. bacillaris*, using 10 informative microsatellite loci, revealed a high degree of genetic heterogeneity [38] (Figure 1). In this study, genotypic characterization of 157 strains from various enological regions (i.e., 28 vineyards/wineries of France, Greece, Hungary, Italy, Spain, Switzerland, and New Zealand), as well as 6 strains from nature, revealed that populations isolated from winemaking environments are quite diverse [38]. Interestingly, neither clonal-like behavior nor specific genetic signatures were associated with strains isolated from different vineyards and wineries, the genetic diversity of *S. bacillaris* strains being shaped by geographical localization [38]. A further study involving the same 163 *S. bacillaris* strains, plus 127 strains isolated from *V. vinifera* and *V. labrusca* ecosystems of.

Argentina (Colonia Caroya, Córdoba) [11,12] and Portugal (Azores Archipelago) [4,5], reinforced the impact of geographic localization on *S. bacillaris* genetic population structure. This study also showed that Argentinian *S. bacillaris* populations are more differentiated from European populations than *S. bacillaris* populations within Europe [37]. In addition, no evidence of genetic differentiation based on the *Vitis* species or vintages, nor an evolving *S. bacillaris* population during alcoholic fermentation was found [37]. Overall, no genetic

signature of *S. bacillaris* strains was found associated with different vintages, *Vitis* species, vineyards, and/or wineries, indicating that winemaking-related factors (i.e., *Vitis* species, vintage, alcoholic fermentation, and/or wineries) do not impact *S. bacillaris* population structure [37,38]. Thus, *S. bacillaris* is not under selective pressure in winemaking environments, representing an interesting model of a non-domesticated ubiquitous wine yeast species [37].

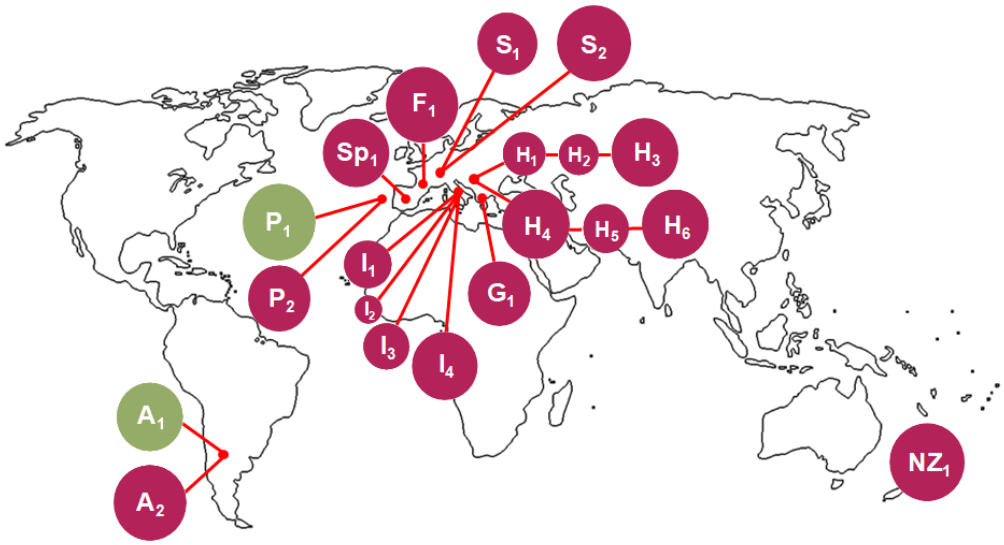

Figure 1. Worldwide diversity of *S. bacillaris* indigenous isolates. Genotypic studies of indigenous *S. bacillaris* strains show rich geographical diversity in Argentina (A_1 and A_2) [37]; France (F_1) [38]; Greece (G_1) [38]; Hungary (H_1, H_2, H_3, H_4, H_5, and H_6) [16]; Italy (I_1) [10], (I_2) [26], (I_3) [23], and (I_4) [38]; New Zealand (NZ_1) [38]; Portugal–Azores Archipelago–(P_1 and P_2) [37]; Spain (Sp_1) [38]; and Switzerland (S_1 and S_2) [16]. *S. bacillaris* isolates from *V. vinifera* and *V. labrusca* are indicated with magenta and green colors, respectively. The areas of the circles are proportional to the *S. bacillaris* strain diversity found in each study (i.e., higher area indicates higher diversity). *S. bacillaris* strains were identified from different sets of indigenous isolates using different molecular genotyping strategies. Strains over total isolates (strains/isolates) as well as their origin and molecular strategy used for identification were: 57/70 (A_1) and 39/40 (A_2) isolates from *V. labrusca* and *V. vinifera* musts, respectively, using PCR amplification of 10 microsatellite loci; 79/84 (F_1) isolates from grape musts and fermenting grape musts, using PCR amplification of 10 microsatellite loci; 15/21 (G_1) isolates from fermenting grape musts, using PCR amplification of 10 microsatellite loci; 2/6 (H_1), 2/7 (H_2), and 5/6 (H_3) isolates from botrytized must, using RAPD-PCR amplification with primers 24, 128, and RF2; 5/6 (H_4), 3/7 (H_5), and 5/6 (H_6) isolates from wine, using micro/minisatellite amplification with primers M13 and $(GTG)_5$; 6/15 (I_1) isolates from Vino Cotto, using RAPD-PCR amplification with primer M13; 9/63 (I_2) isolates from grapes, using *Sau*-PCR amplification with primers SAG1 and SCA and Rep-PCR amplification with primer $(GTG)_5$; 14/36 (I_3) isolates from grapes, using *Sau*-PCR amplification; 14/18 (I_4) isolates from musts, using PCR amplification of 10 microsatellite loci; 5/5 (NZ_1) isolates from fermenting musts, using PCR amplification of 10 microsatellite loci; 13/13 (P_1) and 3/4 (P_2) isolates from *V. labrusca* grapes and *V. vinifera* musts, respectively, using PCR amplification of 10 microsatellite loci; 2/3 (S_1) isolates from botrytized must, using micro/minisatellite amplification with primers M13 and $(GTG)_5$; 3/3 (S_2) isolates from wine, using RAPD-PCR amplification with primers 24, 128, and RF2; and 12/15 (Sp_1) isolates from grape musts and fermenting musts, using PCR amplification of 10 microsatellite loci.

Table 1. Digestion pattern of *S. bacillaris* ITS-5.8S region.

Restriction Enzyme	Fragment Size (bp)		
	S. bacillaris	***C. stellata***	***S. bombicola***
Uncut	460	468	467
*Cfo*I	56 + 103 + 105 + 196	56 + 200 + 212	39 + 56 + 172 + 200
*Hae*III	460	468	54 + 87 + 138 + 326
*Hinf*I	225 + 235	229 + 239	8 + 227 + 232
*Dra*I	309 + 115 + 36	119 + 349	143 + 324
*Mbo*I	22 + 145 + 293	22 + 135 + 149 + 162	22 + 137 + 138 + 170

3. *S. bacillaris* Physiology

S. bacillaris grows as ellipsoid to elongated (2.2–3.0 mm × 3.0–5.2 mm) cells, which divide by multilateral budding [9] (Figure 2). Indigenous strains of this yeast species have been isolated worldwide, from grapes and grape musts, using the general yeast growth media YPD-agar (Figure 3a), the differential media WL-nutrient-agar (Figure 3b), and the selective media YPD agar, supplemented with cycloheximide (Figure 3d–f) and/or lysine-agar [20,48]. In standard YPD-agar media, *S. bacillaris* form small, white, creamy, shiny colonies (Figure 3a), while similar colonies, but green with a white peripheral halo, develop in WL-nutrient agar media (Figure 3b,c), which enables it to be differentiated from other non-*Saccharomyces* species (Figure 3c). Enological species of the *Hanseniaspora* genera (i.e., *H. opuntiae*, *H. osmophila*, *H. uvarum*, and *H. vineae*) also form green colonies in WL-nutrient agar, although these are larger and flat (see Figure 3c).

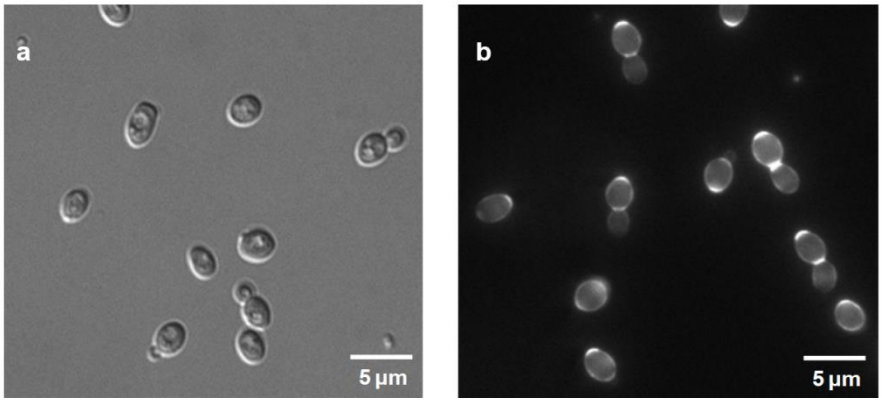

Figure 2. *S. bacillaris* cell morphology. Cellular morphology of *C. zemplinina* (syn., *S. bacillaris*) strain CBS 9494 as observed under differential interference contrast (DIC) microscopy (**a**) or fluorescence (i.e., calcofluor-white staining) microscopy, which highlights cell walls and budding scars (**b**). Images were obtained using a Leica DMI8 inverted microscope and 63× objective.

S. bacillaris can ferment glucose, sucrose, fructose, and raffinose, but not galactose, maltose, and lactose [9]. It shows a marked preference for fructose over glucose when both sugars are present simultaneously [7,10,25,50,51]. This fructophilic character is also associated with other yeast species found in high sugar environments (e.g., *Candida apicola*, *Candida magnoliae*, *Candida versatilis*, *S. bombicola*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces rouxii*) [10,30,52]. Genetic evidence indicates that the fructophilic character of members of the W/S clade is dependent on a specific, low affinity, high capacity fructose transporter named "Ffz1" [52]. In silico analyses of *S. bacillaris* draft genome sequences showed the presence of two distinct *FFZ1* genes at a distance of approximately 4 kb from each other [52]. These transporters (i.e., Ffz1a and Ffz1b) proved to enable growth on fructose and mannose when expressed as sole hexose transporters in a *S. cerevisiae* hxt-null mutant strain [52]. Kinetic parameters of these two transporters revealed that they are not functionally identical: Ffz1a more closely resembles the Ffz1 transporter from *Z. rouxii*,

which seems to be indispensable for fructophily [53], while Ffz1b supports weaker growth on fructose and supports growth on mannose [52].

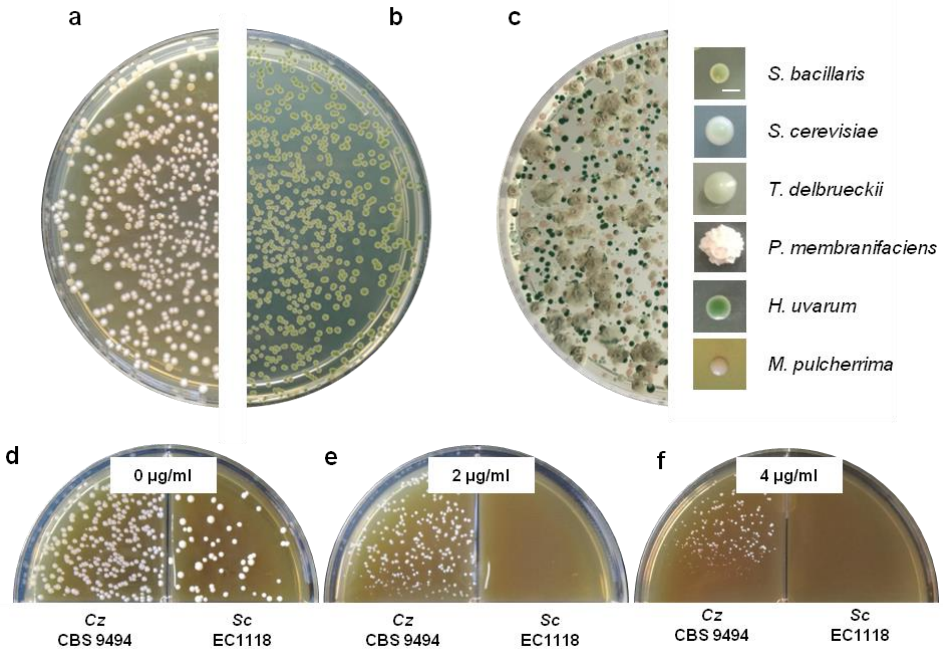

Figure 3. *S. bacillaris* colony morphology. Colonies of *C. zemplinina* (syn., *S. bacillaris*) strain CBS 9494 as observed in standard YPD (**a**) or differential WL-nutrient agar media (**b**), after incubation during 72 h at 25 °C. *S. bacillaris* forms small pale green, creamy colonies in WL-nutrient agar (**b**). "Multi-species" reconstitution experiment of common non-*Saccharomyces* yeasts (*Hanseniaspora uvarum*, *Pichia membranifaciens*, *Metschnikowia pulcherrima,* and *Torulaspora delbrueckii*), *S. cerevisiae* (strain EC1118), and *C. zemplinina* (strain CBS 9494) (**c**). The photography shows the particular morphology and color aspects, on WL-nutrient agar, of the various plated yeast species [49]. Bar in (**c**) represents 1 mm. Identical magnification for all colonies is shown. *S. bacillaris* (*C. zemplinina* CBS 9494; *Cz*) is resistant to cycloheximide (**d**–**f**), a phenotype that allows selective recognition of indigenous non-*Saccharomyces* compared to cycloheximide-sensitive *S. cerevisiae* strains (*Sc*) [20].

An interesting finding concerning fermentation in members of the W/S clade is the apparent absence of a typical pyruvate decarboxylase (PDC) enzyme [30]. In fact, decarboxylation of pyruvate to acetaldehyde is a key step in the alcoholic fermentative pathway, catalyzed by Pdc1 in *S. cerevisiae* [54–56]. Although orthologs of this gene appear to be absent in W/S-clade genomes, a modification of specificity in the enzyme Aro10, allowing this enzyme to accept pyruvate in addition to phenylpyruvate as a substrate, appears to be involved in the remodeling of alcoholic fermentation in W/S clade yeasts [30]. Furthermore, phylogenetic and kinetic analyses of putative alcohol dehydrogenase proteins, Adh1 and Adh6, present in the genomes of W/S-clade species, revealed that the corresponding *ADH1* and *ADH6* genes seem to have been horizontally transferred from bacteria [30]. Ethanol production in members of the W/S clade, conducted by alcohol dehydrogenases of bacterial origin, allow W/S clade species to maintain redox homeostasis (i.e., NAD+ regeneration) when growing under anaerobic conditions [30]. *S. bacillaris*, in particular, harbors one copy of an *ADH1* xenolog; the *ADH6* xenolog was apparently duplicated several times, as this yeast species harbors four *ADH6* paralogs [30].

S. bacillaris uses higher sugar quantities (i.e., up to 40 g/L) than *S. cerevisiae* to produce 1% (*v*/*v*) ethanol [7,23,50,57]. This low ethanol yield, in addition to its low acetic acid production, of *S. bacillaris* compared to *S. cerevisiae*, reveals low activity of the acetaldehyde pathway in *S. bacillaris*, leading to a redistribution in the fluxes of the central carbon metabolism network [58]. Differently from other members of the W/S clade, which convert fructose directly into mannitol [31], *S. bacillaris* overproduces glycerol to maintain the

NADH/NAD+ redox balance in the cells [31,58]. Thus, high glycerol levels are frequently reported for fermentations involving this yeast species [17,58–61]. Interestingly, different levels of glycerol production may be associated with alternative alleles of the *GPP1* gene, encoding glycerol-3-phosphate phosphatase [29].

A reduced lag phase has been observed in *S. bacillaris* strains growing under low nitrogen conditions, suggesting a limited nitrogen requirement of this yeast species [51]. A preferential uptake of ammonium, tryptophan, and arginine, versus other poorly assimilated amino acids, has been observed in fermenting *S. bacillaris* cells [62]. The consumption of nitrogen sources by *S. bacillaris* revealed the strong inability of this species to take up most amino acids in the presence of ammonium [62]. However, nitrogen provided as ammonium versus a mixture of amino acids showed that organic nitrogen compounds supported more efficiently the growth of *S. bacillaris* [62].

Extracellular enzymes produced by *S. bacillaris* include β-glucosidase [50], proteases [26,50], and chitinases [26]. No pectinase, xylanase, lipase, or cellulase activities have yet been reported. The production of these enzymes may be finally dependent on the analyzed strain, the composition of the growing media, and/or the growth conditions [50]. For specific fermentation processes, it may be important to perform a detailed characterization of the extracellular enzymes secreted by *S. bacillaris*, to determine the final chemical profile of wines and/or to use this species as a biocontrol agent [26,27].

Subtle but significant differences have been observed for the various metabolic fermentative traits of *S. bacillaris* strains [16,17,50]. Fermentation vigor, tolerance to ethanol and acetic acid, and H_2S production have been reported as more diverse than ethanol production [16,17,50]. In addition, these differences are affected by abiotic (e.g., nutrient availability, pH, oxygen levels, and temperature) and biotic (e.g., initial cell density and presence of other yeast species) factors [16,63]. Thus, as a warning, genetic similarities found among strains, following genotypic characterization, do not necessarily imply physiological similarities in *S. bacillaris*, and this should be taken into consideration when analyzing genotypic and phenotypic profile correlations [16,17].

4. Industrial Application of *S. bacillaris*

Non-*Saccharomyces* yeast co-starters offer enological advantages compared with single *S. cerevisiae* inoculations [64], contributing to more diverse organoleptic profiles of wines [60,65–70]. Due to its great genetic biodiversity and peculiar metabolism, *S. bacillaris* has been included as a potential co-starter yeast species for industrial mixed fermentations [60,65,71,72].

4.1. S. bacillaris as a Co-Starter in Grape Must Fermentations

In mixed alcoholic fermentations, *S. bacillaris* preferentially consume fructose, providing the evolving *S. cerevisiae* cell population with the use of glucose at both middle and later fermentation stages [60,73]. Thus, co-inoculation of *S. bacillaris* and *S. cerevisiae* strains can result in complete fermentation of the major sugars present in musts [59,63,73]. Moreover, mixed fermentations with *S. bacillaris* can also alleviate osmotic stress for the prevailing *S. cerevisiae* cells, improving fermentation kinetics and reducing acetic acid production [8,65,73,74]. *S. bacillaris* strains can also reduce the final contents of malic acid in wines [6,72,75,76]. This phenomenon appears to be dependent on the use by *S. bacillaris* of malic acid and/or on the stimulation of the malolactic activity of *O. oeni*, thus playing an indirect role in driving malolactic fermentation [75]. It has also been reported, however, that the inoculation of a *S. bacillaris* strain inhibited malolactic fermentation, possibly by the presence of inhibitory compounds that negatively affected the yeast-bacteria interaction [77].

S. bacillaris can normally maintain relatively high cell population levels up to the middle [25,60,78] or even to the final [73,79] stages of fermentation. This may have negative and/or antagonistic consequences of *S. bacillaris* in *S. cerevisiae* growth. In fact, mixed inoculations of *S. cerevisiae* with *S. bacillaris* can lead to a reduction in maximum *S. cerevisiae*

populations in comparison with *S. cerevisiae* single starter fermentations [59,60,63,80]. This could be related to a decrease in nutrient concentrations in the must [25,60,78]. *S. bacillaris* death in mixed inoculated fermentations has also been investigated. Englezos et al. [79] demonstrated that high ethanol concentrations (~11.4% *v*/*v*) did not influence viability loss of *S. bacillaris*. What is more, it has been shown that *S. bacillaris* strains were able to grow at ethanol concentrations as high as 14% *v*/*v*, which could contribute to the successful implantation and good performance of this species during fermentations [10,17,50]. Other *S. bacillaris* isolates were reported to have low tolerance to alcohol levels (up to 5% *v*/*v* ethanol) [11,12]. Because the production of ethanol and other toxic metabolites by *S. cerevisiae* (such as killer toxins, SO_2, and short- to medium-chain fatty acids) have not resulted in a negative co-existence of *S. bacillaris'* populations [11,78], cell-to-cell contact mechanisms may be associated with *S. bacillaris* cell death [78]. Finally, it should be stressed that all reported *S. bacillaris* and *S. cerevisiae* interactions could be strain-specific more than species-specific [25,59].

4.2. S. bacillaris and the Reduction in Ethanol Levels in Wines

Due to its low ethanol yield, *S. bacillaris* is a promising yeast species to reduce ethanol contents in wines [25,50,57]. In recent years, for social, industrial, marketing, and health-associated reasons [81], there has been an increasing interest in reducing the final ethanol concentration of wines. With this aim, different technological and microbiological approaches, including the use of non-*Saccharomyces* starters, have been proposed [17,26,61,72]. Mixed culture fermentations of *S. bacillaris* and *S. cerevisiae* are normally differentiated from *S. cerevisiae* pure culture fermentations because of poor ethanol yields and high glycerol contents [25,50,51,59]. Interestingly, glycerol levels higher than 15 g/L have a positive effect on wine quality and sensory perception by contributing to wine structure and body perception [58].

4.3. Chemical Complexity of S. bacillaris and S. cerevisiae Fermented Beverages

Different complexities of fermented products can be obtained when performing single versus combined *S. cerevisiae* and/or *S. bacillaris* fermentations. Several authors have shown that, in laboratory scale fermentations, the concentrations of some aromatic compounds decline when using *S. bacillaris* and *S. cerevisiae* co-inoculums versus *S. cerevisiae* monocultures (Table 2) [27,58,60,63,65,82]. Other authors, however, have found an increase in volatile compounds when using mixed *S. bacillaris* and *S. cerevisiae* versus single yeast species fermentations [59]. *S. bacillaris* has been reported to overproduce compounds such as H_2S, acetoin, ethyl acetate, and terpenes, which may have a negative impact on the wine organoleptic profile [23,49,61,63]. The apparent contradictory results regarding the sensory characteristics and chemical complexity of these studies could be dependent on: (i) the use of different *S. bacillaris* and *S. cerevisiae* strains, (ii) the inoculation procedures (i.e., simultaneous or sequential inoculations), (iii) the fermentation conditions (i.e., inoculum density, temperature, SO_2, nitrogen and ethanol levels), and/or (iv) the grape must varieties analyzed [63,80,83] (Table 2). In some cases, these outcomes could be the consequence of either negative metabolic or synergistic interactions between *S. bacillaris* and *S. cerevisiae* strains [60,63,83]. Thus, a well-characterized set of co-starter strains and a proper design of the co-fermentations are essential factors to enhance or reduce the presence of particular metabolites [61,64,70]. Under these ideal co-fermentation conditions, the final wines would mimic the organoleptic profile of beverages obtained by spontaneous fermentations, where the local and/or regional sensorial identity of wines is enhanced [61,65,68].

Table 2. Volatile metabolites in *S. bacillaris* and *S. cerevisiae* mixed fermentations.

Fermentation	*S. cerevisiae*	*S. bacillaris*	Inoculation Protocol	Metabolites (Σ)							Ref.
				Alcohols	Fatty Esters	Fatty Acids	Terpenes and C13 Norisoprenoids	lactones	Acetate Esters	Other	
C. sauvignon [1]	Uvaferm BC	FC54	S-24	≈	↓	↓	≈	-	↓	≈	[58]
			S-48	≈	↓	↓	≈	-	↓	≈	
Merlot [1]			S-24	≈	↓	↓	≈	-	↓	≈	
			S-48	≈	↓	↓	≈	-	↓	↓	
Pinot noir [1]			S-24	≈	↓	↓	≈	-	↓	≈	
			S-48	↓	↓	↓	≈	-	↓	↓	
Shiraz [1]			S-24	≈	↓	↓	↑	-	↓	≈	
			S-48	≈	↓	↓	↑	-	↓	≈	
Chardonnay [1]			S-48	≈	↓	↓	↑	-	↓	≈	[83]
Muscat [1]			S-48	≈	↓	↓	↓	-	↓	≈	
Riesling [1]			S-48	≈	≈	↓	≈	-	↓	≈	
Sauvignon blanc [1]			S-48	↑	↑	↓	↑	-	↓	≈	
Golden delicious [2]	EC1118	CHIAR4	S-48	↓	↓	↓	↑	-	↓	↑	[27]
		PECO4	S-48	↓	↓	↓	↑	-	↓	↑	
Sauvignon blanc [1]	PB2023	MCR-9	Co	≈	↓	≈	↑	≈	↓	↓	[63]
K&M [1,3]	SacPK7	StbPK9	Co	≈	≈	≈	≈	-	≈	≈	[60]
			S-23	≈	≈	≈	≈	-	≈	≈	
M [4]	SRS1	STS12	Co	↓	≈	≈	≈	-	-	≈	[65]
Macabeo	QA23	CszB4	Co	↑	↑	↓	-	-	≈	-	[82]

[1] Grape must; [2] apple juice; [3] Kotsifali and Mandilari; [4] Montepulciano d'Abruzzo; either significant ↑: increase or ↓: decrease, with respect to control fermentation with *S. cerevisiae* control strain; -: not determined; ≈: not significant; S-: sequential inoculation (i.e., either 23, 24, or 48 h); Co: co-inoculation.

Concerning the production of acetate and ethyl fatty esters, contradictory results have been obtained when using *S. bacillaris* in mixed fermentations (Table 2). In fact, mixed inoculations may result in increased [60,65,82,83] or reduced [58,63] overall levels of the various esters analyzed. Similar results were observed when analyzing higher alcohols. In these studies, *S. bacillaris* has been associated with either increased levels of total [82,83] and specific [58,59] or reduced [27,65] overall levels of these compounds (Table 2). Genome comparisons between two *S. bacillaris* strains (i.e., PAS13 and FRI751) and *S. cerevisiae* strain EC1118 revealed that the *S. bacillaris* branched-chain amino acid aminotransferase (BCAT) enzyme was strongly divergent from that of *S. cerevisiae* [29]. These differences in BCAT enzymes could influence valine, leucine and isoleucine degradation, and potentially the corresponding higher alcohol productions [29]. In the case of other aromatic compounds, like terpenes and C-13 norisoprenoids, their presence and relative concentration levels are related to the fermentation matrix (i.e., fruits and/or fruit varietals) and strain tested [27,58,63,83]. Again, either an increase or no change in their presence and relative levels was observed in *S. cerevisiae* and *S. bacillaris* mixed fermentations using alternative grape varietals and fruits and/or yeast strains (Table 2).

Production of H_2S by *S. bacillaris* seems to also be variable and strain-specific. Different authors have reported high [17], medium [12,17,23], and low [23,84] H_2S production from *S. bacillaris* strains. In some studies, the temperature of the fermentations has been considered, because production of H_2S seems to increase at higher temperatures [50]. Mixed inoculations using *S. cerevisiae* and *S. bacillaris* result in wines with higher levels of sulfur compounds [74]. Other undesirable compounds, like volatile fatty acids, showed a reduction in mixed fermentations with *S. cerevisiae* and *S. bacillaris* [58,82,83]. Low production of acetic acid by *S. bacillaris* strains, either in pure or in mixed fermentations, has been reported [6,17,23,25,51,60,61,63,74]. Other *S. bacillaris* strains, however, have been shown to produce relatively high levels of acetic acid [50].

4.4. Biocontrol Potential of S. bacillaris

S. bacillaris strains have been studied as a safe and eco-friendly method to control several diseases affecting fruit crops and their associated products [26,27]. Lemos Junior et al. (2020) reported the absence of pathogenicity factors for human health of *S. bacillaris* strains, including growth at 37 °C, pseudohyphae formation, invasive growth, and proteolytic activity, which guarantees that these strains do not represent a risk for human health [27]. Even when *S. bacillaris* has been associated with table grape sour rot [85], the use of *S. bacillaris* may control several fungal diseases and may also present a potential positive impact on subsequent fermentations [26,27].

The biocontrol activity of selected *S. bacillaris* strains has been studied against the gray mold disease agent, *Botrytis cinerea*, in apples and grapes [26,27]. These studies showed that the possible antifungal mode of action of this species is volatile organic compound (VOC) production, which, in turn, present inhibitory effects both in vivo and in vitro [26,27]. VOCs are suggested as the main compounds responsible for the reduction in fungal radial mycelial growth and *B. cinerea* gray mold decay [26,27], possibly due to the antimicrobial action of benzyl alcohol. In addition, *Alternaria alternata* grape infections and toxin production [86] have been successfully controlled by the use of *S. bacillaris* strains. The biocontrol of *A. alternata* could be the result of *S. bacillaris*' ability to colonize wound sites, which implies competitive mechanisms [86].

5. Conclusions

Starmerella bacillaris (syn., *C. zemplinina*) is a fructophilic non-*Saccharomyces* yeast species ubiquitously present in grapes, grape musts, and flowers. Surprising recent findings concerning the genetic diversity and metabolism of *S. bacillaris* have positioned this yeast species as an important model microorganism for evolutionary and metabolic studies, as well as the potential industrial and biocontrol uses. Detailed population genetic analyses of the *S. bacillaris* species, and comparative genomic studies in the genera *Starmerella*,

have revealed the rich diversity of *S. bacillaris* worldwide, as well as the existence of complex HGT events that have exquisitely redesigned some metabolic pathways. These observations open a path for further studies on ecological and evolutionary aspects of the metabolism in *S. bacillaris*. Finally, the selection of unique, enologically advantageous *S. bacillaris* co-starter strains showing desired fermentation profiles would contribute to satisfy winemakers' and the consumer's expectations.

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