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Influence of Single Nitrogen Compounds on Growth and Fermentation Performance of *Starmerella bacillaris* and *Saccharomyces cerevisiae* during Alcoholic Fermentation

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ABSTRACT Nitrogen is among the essential nutriments that govern interactions between yeast species in the wine environment. A thorough knowledge of how these yeasts assimilate the nitrogen compounds of grape juice is an important prerequisite for a successful co- or sequential fermentation. In the present study, we investigated the efficiency of 18 nitrogen sources for sustaining the growth and fermentation of two *Starmerella bacillaris* strains displaying metabolic properties, compared to the reference yeast *Saccharomyces cerevisiae*. The analysis of growth and fermentation parameters provided a comprehensive picture of *S. bacillaris* preferences with respect to nitrogen sources for sustained growth and fermentation. Important differences were observed with *S. cerevisiae* regarding rates, final population, and CO_2 production. In particular, Lys and His supported substantial *S. bacillaris* growth and fermentation contrary to *S. cerevisiae*, while only 3 nitrogen sources, Arg, NH_4^+, and Ser, promoted *S. cerevisiae* growth more efficiently than that of *S. bacillaris* strains. Furthermore, *S. bacillaris* strains displayed a higher fermentative activity than *S. cerevisiae* during the first phase of culture with Gly or Thr, when the former species consumed solely fructose. Finally, no correlation has been shown between the ability of nitrogen sources to support growth and their fermentation efficiency. The specificities of *S. bacillaris* regarding nitrogen source preferences are related to its genetic background, but further investigations are needed to elucidate the molecular mechanisms involved. These data are essential elements to be taken into account in order to make the best use of the potential of the two species.

IMPORTANCE Mixed fermentations combining non-*Saccharomyces* and *S. cerevisiae* strains are increasingly implemented in the wine sector, as they offer promising opportunities to diversify the flavor profile of end products. However, competition for nutrients between species can cause fermentation problems, which is a severe hindrance to the development of these approaches. With the knowledge provided in this study on the nitrogen preferences of *S. bacillaris*, winemakers will be able to set up a nitrogen nutrition scheme adapted to the requirement of each species during mixed fermentation through must supplementation with relevant nitrogen compounds. This will prevent nitrogen depletion or competition between yeasts for nitrogen sources and, consequently, potential issues during fermentation. The data of this study highlight the importance of appropriate nitrogen resource management during co- or sequential fermentation for fully exploiting the phenotypic potential of non-*Saccharomyces* yeasts.

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The main characteristic that differentiates grape juice from other yeast habitats is the nitrogen resource, often in growth-limiting concentrations (1) and present as a complex mixture of proteins, peptides, amino acids, and ammonium, of which only a fraction is assimilated by yeasts (referred to as yeast assimilable nitrogen [YAN]). During wine fermentation, YAN plays a central role, influencing both fermentation performances and metabolite production by yeasts. Yeast growth and fermentation kinetics greatly depend on both the quantity and nature of available nitrogen sources (2, 3). Furthermore, some amino acids are precursors for the synthesis of volatile compounds that significantly contribute to wine flavor and aroma (4, 5). As a consequence, the management of the nitrogen resource in terms of both concentration and composition is fundamental to ensure a fast start and a steady, complete fermentation (6). However, implementing these strategies requires an increased knowledge on the operative metabolic nitrogen network and its regulation, inasmuch as the assimilation and further use of nitrogen sources by yeasts may be modulated by many environmental and genetic factors (7–10).

The uptake and metabolism of nitrogen compounds have been widely studied in the model yeast species *Saccharomyces cerevisiae* (9, 11). In particular, on one hand, YAN molecules have been differentiated in different classes on the basis of their efficiency to promote growth when used as the sole nitrogen source by comparing generation times (12, 13). Thus, ammonium, glutamine, and asparagine are considered to be preferred nitrogen sources by *S. cerevisiae*, whereas proline and urea, poorly sustaining growth, are regarded as nonpreferred sources. On the other hand, the assimilation sequence of nitrogen sources provided as a mixture during fermentation has been elucidated (11, 14). The nitrogen molecules were classified into three clusters, prematurely, early, and late consumed, according to their order of uptake during wine fermentation. This sequence is entirely explained by the regulatory mechanisms and kinetic properties of the permeases involved in the transport of nitrogen compounds: the uptake of early consumed amino acids is mediated by specific permeases, induced according to the availability of the transported amino acids via the Ssy1-Ptr3-Ssy5 (SPS) sensor (15, 16); late-consumed amino acids enter cells through general permeases under nitrogen catabolite repression (NCR) or SPS-regulated low-affinity permeases (12, 17). Interestingly, the order of assimilation is not necessarily associated with its efficiency to sustain *S. cerevisiae* growth (2).

Conversely, the physiology and metabolism of non-*Saccharomyces* species and their regulation by environmental conditions have been far less explored, despite their growing use in winemaking. Because of their metabolic and phenotypic specificities, these yeasts offer very promising opportunities to diversify and increase the complexity of the flavor profile of wines and, as a consequence, to meet consumers' expectations in an increasingly competitive market. Thus, in order to improve wine-specific characteristics, mixed fermentations combining non-*Saccharomyces* and *S. cerevisiae* strains are being promoted in the wine sector (18, 19). Indeed, non-*Saccharomyces* species are generally little to moderately tolerant to ethanol stress and, therefore, need to be combined with an *S. cerevisiae* strain to achieve fermentation to dryness (19). As a consequence, competition for nutrients takes place between the two yeast species, in particular regarding nitrogen sources (6), and an in-depth knowledge of nitrogen source assimilation in non-*Saccharomyces* yeasts, unavailable until now, is required for an efficient exploitation of their potentialities during winemaking.

Among non-*Saccharomyces* yeasts, the use of selected *Starmerella bacillaris* (synonym *Candida zemplinina*) strains in mixed culture fermentations with *S. cerevisiae* holds promise as a way to reduce ethanol and increase total acidity and glycerol content in wines (20–22). However, interactions between these species need to be examined in more detail, since they can greatly impact their growth and the wine's final chemical

composition (23). In particular, the impact of *S. bacillaris* in mixed fermentations can be more effective with an appropriate management of the nitrogen resource throughout wine fermentation. In this context, an important question to be addressed and which still remains open is how single nitrogen sources affect growth and fermentation performances in *S. bacillaris* and, as a consequence, metabolite production. With this aim, we investigated in this study how 18 single nitrogen compounds (17 single amino acids and ammonium) affected the growth and fermentation kinetics of *S. bacillaris* and *S. cerevisiae*. The results of this study could help winemakers to optimize alcoholic fermentation, considering the specific nitrogen sources necessary to promote growth in inoculated wine yeasts.

RESULTS

Influence of single nitrogen sources on the growth of each yeast strain. The ability of *S. cerevisiae* and *S. bacillaris* strains to grow using 18 different compounds as the sole nitrogen source was investigated, monitoring growth with a microplate reader during the first 48 h of fermentation at 25°C. Growth parameters—lag phase time (T_{lag} [h]), maximum growth rate (μ_{max} [1/h]), and maximum optical density (OD_{48h})—were compared, and results are presented in Table 1 and Fig. 1 and 2.

The growth patterns in terms of OD evolution over time revealed that the two *S. bacillaris* strains generally behaved similarly, even if some variations were observed either in the maximal growth rate (0.095 and 0.061 h^{-1} for FC54 and MUT5705 on Arg, respectively) or in OD_{48h} (1.32 and 1.22 for FC54 and MUT5705 on Ser, respectively) (Table 1; Fig. 1). Conversely, for most nitrogen compounds used during fermentation, great differences in growth profiles were observed between the two *S. bacillaris* strains on one hand and *S. cerevisiae* on the other hand, resulting in substantial discrepancies in the quantitative growth parameters T_{lag}, μ_{max}, and OD_{48h}. The only exceptions to this general pattern were Ala, Asp, Glu, and Gln, for which the profiles of the 3 strains were very close, with less than 20% variation in their growth parameters. For illustration purposes, during fermentation on glutamate, the lag phase, maximal growth rate, and OD_{48h} ranged between 13.1 and 14.2 h, 0.104 and 0.108 h^{-1}, and 1.34 and 1.44, respectively (Table 1).

Interestingly, only 3 nitrogen sources, Arg, NH_4^+, and especially Ser, promoted *S. cerevisiae* growth more efficiently than that of the *S. bacillaris* strains. As an example, a 44% and 57% increase in the lag phase time was measured during FC54 and MUT5705 fermentation, respectively, on Ser compared with that of *S. cerevisiae* Uvaferm BC combined with a 40% decrease in the maximal fermentation rate. OD_{48h} was not substantially different between strains during fermentation on Ser and Arg, with variations ranging from 5% to 18%, while the OD_{48h} measured during *S. bacillaris* FC54 growth on ammonium was decreased by 43% compared with that of Uvaferm BC.

The second point to be highlighted was that both *S. bacillaris* strains were able to grow on all of the tested nitrogen sources, while Cys, Lys, His, and Gly only poorly sustained or even did not allow *S. cerevisiae* growth with μ_{max} and OD_{48h} values lower than 0.013 h^{-1} and 0.28, respectively. In particular, during fermentation on Lys, *S. bacillaris* strains displayed a 0.107 and 0.085 h^{-1} μ_{max} and OD_{48h} values of 1.54 and 1.45. Regarding the lag phase, moderate (Lys and Cys, between 14.2 and 18.6 h) to long (His and Gly, longer than 26.8 h) durations were found for *S. bacillaris*, depending on the amino acid.

Finally, most of the amino acids catabolized through the Ehrlich pathway, i.e., Leu, Val, Phe, Met, Thr, and Trp, were more easily assimilated by the two *S. bacillaris* strains than by *S. cerevisiae*. Usually, compared to Uvaferm BC, FC54 and MUT5705 exhibited a higher growth rate (3 times higher during Trp fermentations) and a shorter lag phase (−8.7 h and −5.8 h during Trp fermentations, respectively) with some exceptions; no significant differences were found in lag phase duration during Thr fermentation between the 3 strains and in the maximum growth rate during Val fermentation between Uvaferm BC and MUT5705. Finally, the changes in T_{lag} and μ_{max} had no

TABLE 1 Growth parameters measured in wines fermented using a single nitrogen source[a]

	Lag phase time (h)				Maximum growth rate (μ_{max} [1/h])				Maximum OD (OD_{max})			
N source	Uvaferm BC	FC54	MUT5705	Significance[b]	Uvaferm BC	FC54	MUT5705	Significance[b]	Uvaferm BC	FC54	MUT5705	Significance[b]
Ala	14.93 ± 0.35	13.23 ± 0.95	12.71 ± 0.24	NS	0.076 ± 0.004b	0.078 ± 0.001b	0.061 ± 0.001a	**	1.105 ± 0.005b	1.034 ± 0.013a	1.037 ± 0a	**
Arg	9.67 ± 0.43a	13.27 ± 0.69b	11.45 ± 0.13ab	*	0.099 ± 0.001b	0.095 ± 0.002b	0.061 ± 0.001a	***	1.685 ± 0.006c	1.602 ± 0.01b	1.482 ± 0.012a	***
Asp	11.81 ± 0.02a	15.74 ± 0.28c	14.07 ± 0.27b	***	0.105 ± 0.008	0.104 ± 0.002	0.087 ± 0.001	NS	1.456 ± 0.003b	1.346 ± 0.013a	1.376 ± 0.003a	**
Cys	1.12 ± 0.12a	18.58 ± 0.44b	17.79 ± 0.61b	***	0.011 ± 0.001a	0.07 ± 0.008c	0.046 ± 0.003b	**	0.143 ± 0.013a	1.242 ± 0.035c	1.098 ± 0.009b	***
Gln	11 ± 0.33	11.72 ± 0.58	11.53 ± 0.18	NS	0.139 ± 0.001b	0.118 ± 0.002a	0.124 ± 0.004a	**	1.728 ± 0.048b	1.511 ± 0.001a	1.502 ± 0.014a	**
Glu	13.65 ± 0.25	14.22 ± 0.77	13.11 ± 0.39	NS	0.107 ± 0.003	0.104 ± 0.002	0.108 ± 0.001	NS	1.442 ± 0.033b	1.337 ± 0.017a	1.378 ± 0ab	*
Gly	31.86 ± 0.03	34.23 ± 1.13	33.63 ± 0.57	NS	0.011 ± 0.005a	0.044 ± 0.01b	0.055 ± 0.004b	*	0.275 ± 0.078a	0.765 ± 0.078b	0.955 ± 0.076b	**
His	1.37 ± 0.26a	26.58 ± 2.52b	29.03 ± 2.02b	**	0.01 ± 0.001a	0.056 ± 0.006b	0.048 ± 0.001b	**	0.28 ± 0.042a	1.135 ± 0.007b	0.945 ± 0.078b	***
Leu	23.42 ± 0.01b	14.63 ± 0.43a	14.21 ± 0.54a	***	0.055 ± 0.001a	0.1 ± 0.006c	0.085 ± 0.001b	**	1.26 ± 0a	1.335 ± 0.035b	1.345 ± 0.007b	*
Lys	1.42 ± 0.05a	15.77 ± 0.97b	14.23 ± 0.13b	***	0.013 ± 0.001a	0.107 ± 0.001c	0.085 ± 0.001b	***	0.17 ± 0.014a	1.535 ± 0.007c	1.455 ± 0.007b	***
Met	17.96 ± 0.23	16.69 ± 0.81	15.82 ± 0.86	NS	0.051 ± 0a	0.09 ± 0.008b	0.074 ± 0.006ab	*	1.285 ± 0.007	1.29 ± 0.042	1.27 ± 0.014	NS
Phe	19 ± 0.06c	16.18 ± 0.4b	14.6 ± 0.08a	***	0.058 ± 0.003a	0.098 ± 0.006b	0.068 ± 0.001a	**	1.31 ± 0.014b	1.28 ± 0ab	1.265 ± 0.007a	*
Pro	22.9 ± 1.58b	15.53 ± 0.26a	14.94 ± 0.29a	**	0.054 ± 0.013a	0.093 ± 0.008b	0.089 ± 0.002ab	*	1.24 ± 0.085a	1.355 ± 0.007b	1.375 ± 0.007b	**
Ser	14.82 ± 0.47a	21.31 ± 1.61b	23.35 ± 0.37b	**	0.105 ± 0.001b	0.064 ± 0.001a	0.064 ± 0.002a	***	1.43 ± 0.014b	1.32 ± 0.014a	1.22 ± 0.014a	**
Thr	27.61 ± 3.77	25.95 ± 1.46	29.62 ± 0.09	NS	0.022 ± 0.005a	0.059 ± 0.003b	0.058 ± 0.002b	**	0.55 ± 0.028a	1.285 ± 0.007b	1.235 ± 0.086b	***
Trp	25.66 ± 0.88c	16.98 ± 0.48a	19.84 ± 0.09b	**	0.026 ± 0.001a	0.085 ± 0.001b	0.084 ± 0b	***	0.695 ± 0.007	1.42 ± 0.028	1.415 ± 0.021	***
Val	24.65 ± 0.41b	16.44 ± 1.36a	15.11 ± 0.45a	**	0.07 ± 0.001a	0.099 ± 0.003b	0.07 ± 0.008a	*	1.36 ± 0	1.34 ± 0.028	1.33 ± 0.014	NS
$NH4^+$	9.11 ± 0.94b	5.7 ± 0.86a	5.99 ± 0.1a	*	0.034 ± 0.001b	0.022 ± 0.004a	0.02 ± 0.002a	*	0.56 ± 0.057b	0.32 ± 0.014a	0.485 ± 0.064ab	*

[a]Growth parameters (lag phase time, maximum growth rate [μ_{max}], and maximum OD [OD_{max}]) of the different *Saccharomyces cerevisiae* and *Starmerella bacillaris* strains in SM200 with a single nitrogen source. Uvaferm BC, *S. cerevisiae*; FC54 and MUT5705, *S. bacillaris*. Different letters within the same row indicate significant differences among the different strains used according to the Tukey-HSD test ($P < 0.05$).

[b]*, **, ***, and NS indicate significance at *P* values of <0.05, <0.01, <0.001, and not significant, respectively.

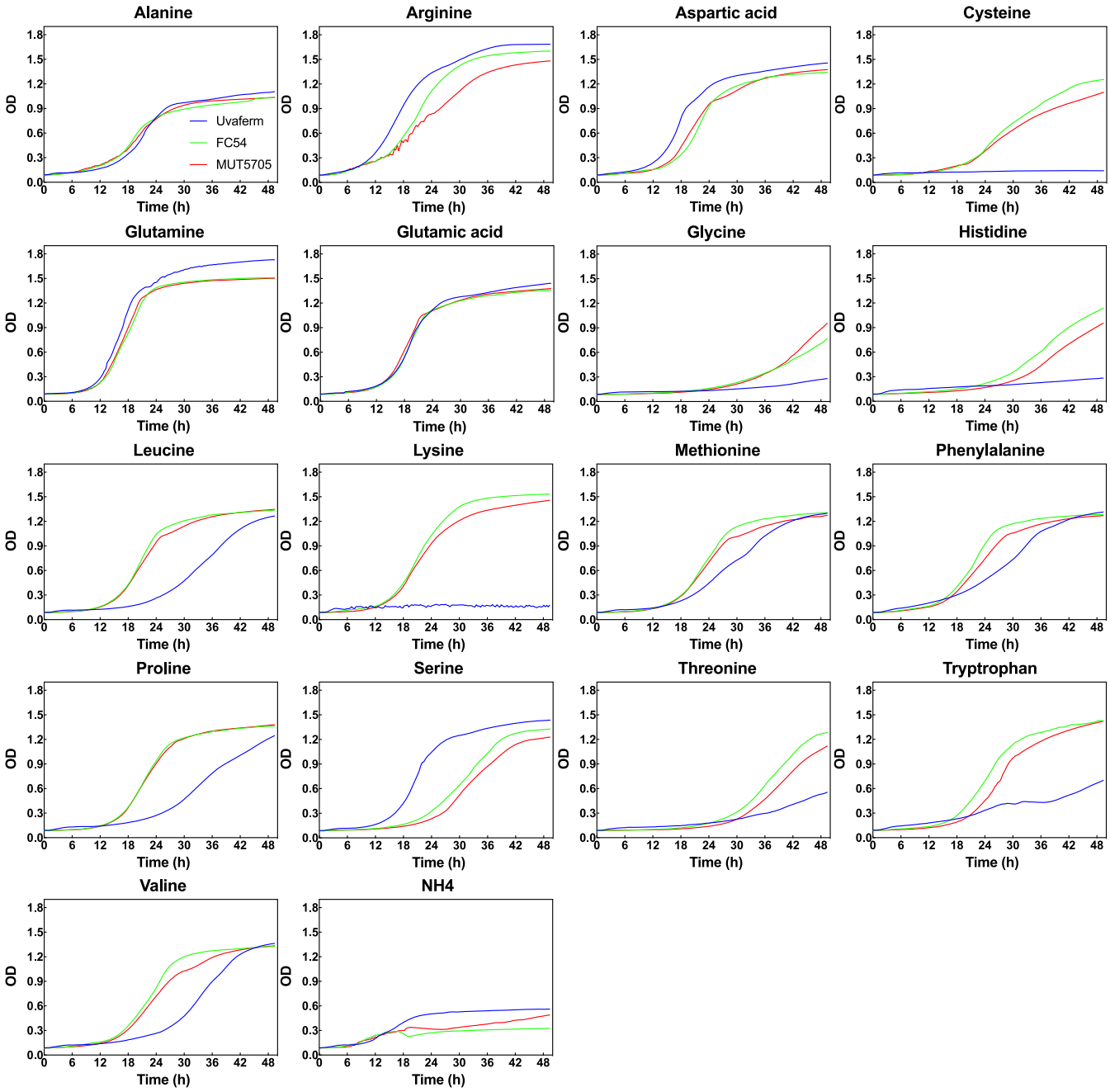

FIG 1 Effect of single nitrogen sources on the optical density (OD) evolution of the strains tested. For each strain, experimental data are shown as indicated in the symbol key. Mean values are calculated from 2 replicates.

significant incidence on OD_{48h} apart from fermentation on Thr and Trp, in which *S. bacillaris* strains displayed values 2× higher than that of *S. cerevisiae*. To sum up, box plots providing an overview of the performances of the 3 strains under different nitrogen conditions (Fig. 2A) revealed that, according to the nitrogen source, both *S. bacillaris* strains displayed a lower variability than *S. cerevisiae* in terms of μ_{max} and OD_{48h}. This observation reflected the capacity of *S. bacillaris* strains to use more efficiently a larger range of nitrogen sources for growth compared to *S. cerevisiae*.

To further investigate these differences, we compared, for each species, nitrogen source efficiency in supporting growth according to the classification previously defined by Cooper (12) and Godard et al. (13) on the basis of growth parameters. Drawing up heat maps from the data sets revealed that the nitrogen compounds

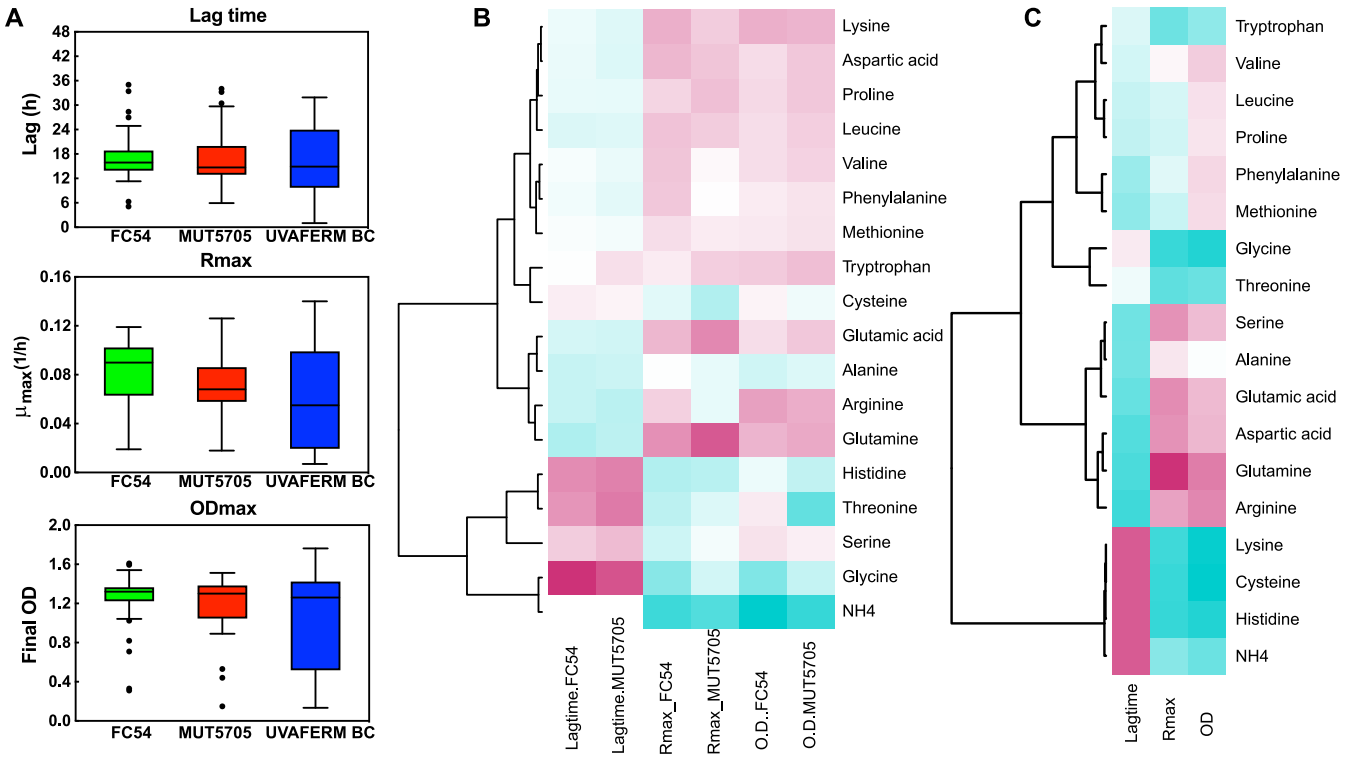

FIG 2 (A) Box plots display the distribution of all of the nitrogen sources for each strain according to their performance in lag phase, R_{max}, and final optical density. (B) Growth parameters of the 2 *Starmerella bacillaris* strains grown in synthetic must with only one nitrogen source. (C) Growth parameters of *Saccharomyces cerevisiae* Uvaferm BC in synthetic must with only one nitrogen source. Heatmap of the average linkage hierarchical clustering is based on the Euclidean distance measurements of growth parameters. Each row in the heatmap represents the single nitrogen source used for growth. Colors from blue to red represent values from low to high compared with the mean (white). The cluster separates the nitrogen sources into different clades, which represent poor, intermediate, and good nitrogen sources for the growth of the different strains as previously defined by Cooper (12) and Godard et al. (13).

herein could be clustered into 3 categories for each species, *S. bacillaris* (Fig. 2B) and *S. cerevisiae* (Fig. 2C, used as reference). First, Asp, Gln, Glu, Pro, Arg, Leu, and Lys were classified as preferred amino acids by *S. bacillaris* strains, with growth patterns characterized by a short lag phase (between 11.5 h and 15.5 h), high μ_{max} (from 0.084 h^{-1} to 0.126^{-1}), and OD_{48h} (from 1.34 to 1.60). The intermediate group, consisting of Ala, Met, Phe, Val, and Trp, was differentiated by either a longer lag phase (e.g., in the case of Trp, 17 h and 19.8 h) or a lower growth rate (e.g., in the case of Ala, 0.78 h^{-1} and 0.061 h^{-1}) or a lower OD_{48h} (e.g., in the case of Ala, 1.034 and 1.037). Finally, NH_4^+, Ser, Thr, Cys, Gly, and His were assimilated less efficiently and poorly sustained growth in both FC54 and MUT5705. In these conditions, the growth of the two *S. bacillaris* strains displayed the longest lag phase (ranging from 17.8 h to 34.2 h) together with the lowest μ_{max} (between 0.07 h^{-1} and 0.02 h^{-1}) and, in the case of Gly and NH_4^+, a very low population as show by OD_{48h} values (FC54, 0.76 and 0.32; MUT5705, 0.95 and 0.48).

Interestingly, the two *S. bacillaris* strains showed different growth capabilities compared to those of *S. cerevisiae* in the presence of specific nitrogen compounds. Indeed, a broader range of preferred growth-supporting amino acids was found in *S. bacillaris*. In particular, Leu and Lys clustered in this group together with Asp, Gln, Glu, Pro, and Arg, while in *S. cerevisiae*, Leu only poorly supported growth and Lys was simply not suitable as the sole nitrogen source. Similarly, Trp, which allowed intermediate growth in *S. bacillaris*, did not support *S. cerevisiae* growth. Conversely, Ser clearly appeared as a nonpreferred amino acid for *S. bacillaris*, although it was one of the 4 most efficient nitrogen sources sustaining *S. cerevisiae* growth.

Influence of nitrogen sources on the fermentation performance of each yeast strain. Yeast ability to achieve wine fermentation depends not only on growth capacity but also on other phenotypic traits, such as resistance to ethanol or fructophilic character, which could be modulated according to the nitrogen source. In this context, we

investigated the impact of 18 nitrogen sources on the fermentation performances of *S. bacillaris* FC54 and MUT5705 and *S. cerevisiae* Uvaferm BC (control). The on-line monitoring of fermentative activity enabled the accurate calculation of the main fermentation parameters as follows: maximum CO_2 production (g/liter), maximal fermentation rate (R_{max}) (g/liter/h), time to achieve R_{max} (h), CO_2 production at R_{max} (g/liter), lag phase time (h), time to achieve a 60-g CO_2 production (T_{60g}) (h), fermentation rate measured after production of 60 g CO_2 (R_{60CO2}) (g/liter/h), and the maximal rate measured during the second stage of fermentation ($R_{max2stage}$) (g/liter/h). This 2nd phase was observed only in *S. bacillaris* fermentation profiles, after 144 h of culture, and corresponded to a substantial decrease in the fermentation rate due to fructose exhaustion (Fig. 3).

Focusing on the ability to complete 200 g/liter sugar fermentation, *S. bacillaris* strains achieved lower scores for CO_2 production than *S. cerevisiae*. The reference species was able to achieve fermentation to dryness regardless of the nitrogen source used, as shown by the final CO_2 production, between 93.5 and 97.8 g/liter, apart from Lys, His, Cys, and Trp (Fig. 3). Conversely, *S. bacillaris* strains were not able to complete fermentation, in particular strain FC54, producing no more than 80 g/liter CO_2 whatever the nitrogen source used. For this strain, the lowest CO_2 production was registered during fermentations with Cys, Phe, and Lys (final CO_2, $<$60 g/liter) and Ser, Thr, Gly, Leu, Ala, Arg, Asp, and Trp (final CO_2, $<$70 g/liter). It is worth noticing that *S. bacillaris* MUT5705 displayed greater fermentation capacities, exhausting almost all of the sugars from the medium in the presence of Ser, Pro, His, or Thr (final CO_2 production, $>$94 g/liter), even if CO_2 production in this strain did not exceed 60 g/liter on Cys and Phe and 80 g/liter on Lys, Asp, and Glu.

Important variations were observed in the lag phase, which lasted from under 15 h up to almost 100 h, depending on the species and nitrogen source used (Fig. 4). Generally, *S. bacillaris* strains were characterized by a lower lag phase variability according to the nitrogen source than *S. cerevisiae*. Furthermore, for the majority of nitrogen sources, the lag phase of both *S. bacillaris* strains lasted around 20 h, with some exceptions, namely, Gly, His, and Thr, for which longer phases (about 30 h) were registered. Concerning *S. cerevisiae*, the variability according to the nitrogen source was substantially larger: longer lag phases, over 100 h, were observed during fermentation on Cys, Gly, His, Ile, and Lys, while at the opposite, very short lag phases, shorter than 15 h, were found when *S. cerevisiae* was grown on Asp, Arg, Gln, Glu, NH_4^+, and Ser.

Surprisingly, the profiles of CO_2 accumulation and production rate during fermentation with *S. bacillaris* displayed two separate phases, which are not observed during *S. cerevisiae* fermentations (Fig. 3 and 5). During fermentations performed by *S. bacillaris*, a change in the slope of the CO_2 production curve was observed at a transition point, around approximately 135 h to 150 h of fermentation. This pattern was likely related to the specificity of *S. bacillaris* strains regarding sugar consumption, with a strong fructophilic character (21). The maximal fermentation rate was registered during the 1st phase, where fructose was preferentially consumed by *S. bacillaris*. Interestingly, both strains demonstrated a low variation in their maximal fermentation rate according to the nitrogen source, comprised of values from 0.47 to 0.74 g/liter/h and between 0.56 and 0.85 g/liter/h for FC54 and MUT5705, respectively. In addition, both *S. bacillaris* strains displayed a lower fermentation capacity than *S. cerevisiae* in the presence of most nitrogen sources, for which the maximal fermentation rate varied more largely from 0.43 to 1.91 g/liter/h. However, some exceptions to this general pattern should be pointed out. First, R_{max} comparison revealed that fermentation performances of *S. bacillaris* on His (FC54, 0.69 g/liter/h; MUT5705, 0.71 g/liter/h), Cys (FC54, 0.13 g/liter/h; MUT5705, 0.16 g/liter/h), and Lys (FC54, 0.47 g/liter/h; MUT5705, 0.68 g/liter/h) were greater than *S. cerevisiae* (His, 0.39 g/liter/h; Cys, 0.07 g/liter/h; Lys, 0.17 g/liter/h). Finally, the R_{max} values measured during fermentations on Thr (FC54, 0.56 g/liter/h; MUT5705, 0.69 g/liter/h; Uvaferm BC, 0.61 g/liter/h) and Gly (FC54, 0.54 g/liter/h; MUT5705, 0.51 g/liter/h; Uvaferm BC, 0.62 g/liter/h) were similar for the 3 strains; however, this maximal value was

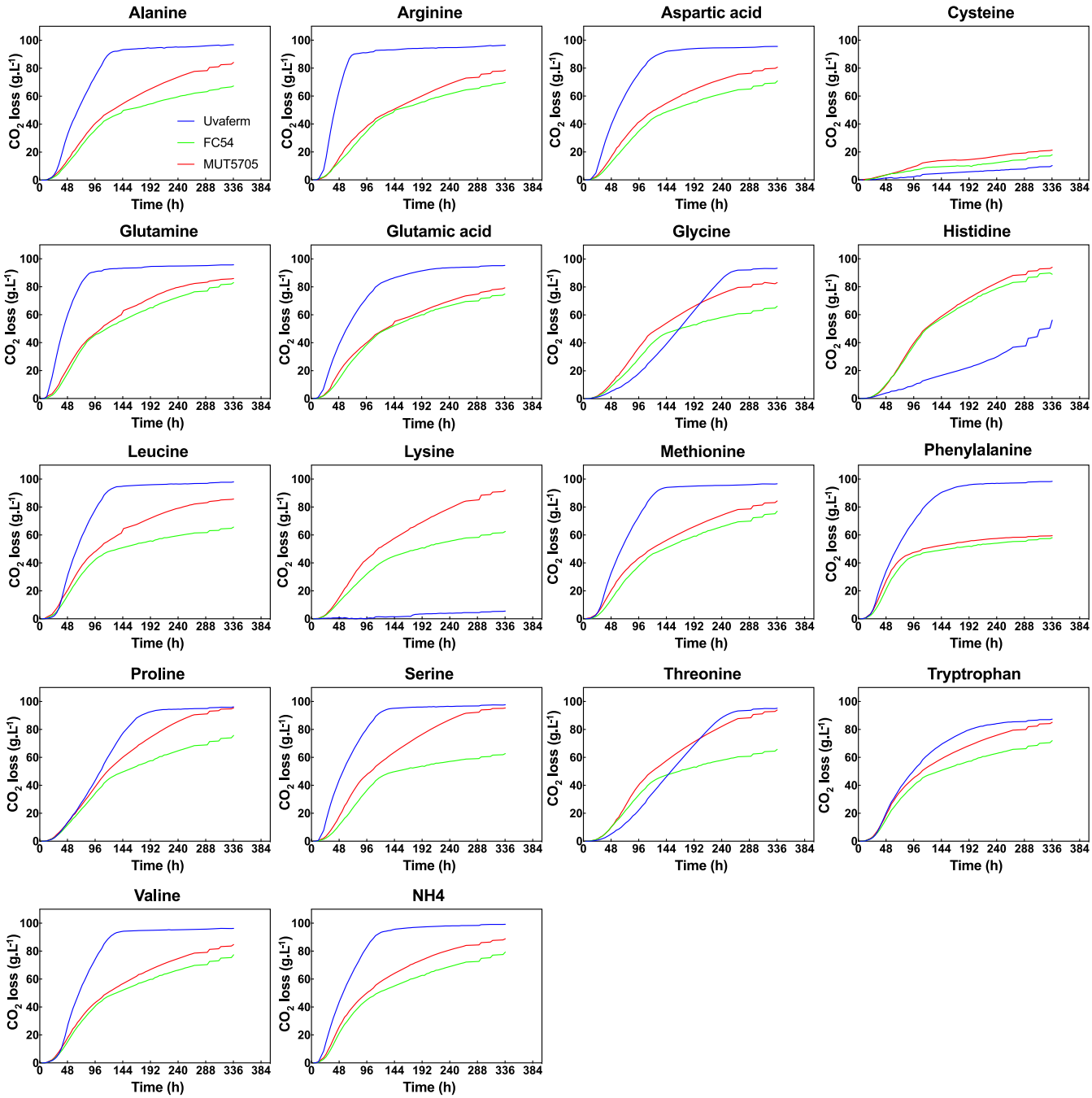

FIG 3 Effect of single nitrogen source on the CO_2 production evolution of the strains tested. For each strain, experimental data are shown as indicated in the symbol key. Mean values are calculated from 2 replicates.

achieved at a late stage of *S. cerevisiae* fermentation (150 to 200 h), while it was already reached during the first phase for *S. bacillaris* strains (Fig. 3).

The 2nd phase observed after 144 h in *S. bacillaris* fermentation profile corresponded to a substantial decrease in the fermentation rate, i.e., when glucose was mainly consumed. The maximal fermentation rates ($R_{max2stage}$) measured during this period ranged from 0.05 g/liter/h to 0.28 g/liter/h and were significantly lower than those achieved in the 1st phase (from 0.47 to 0.96 g/liter/h, apart from Cys), representing reductions from 50% (Cys) to 90% (Phe). The comparison of these rates revealed a wide variability, depending on the strain and nitrogen source. Thus, the lowest $R_{max2stage}$ was registered in the presence of Phe as the sole nitrogen source (0.06 g/liter/h for both

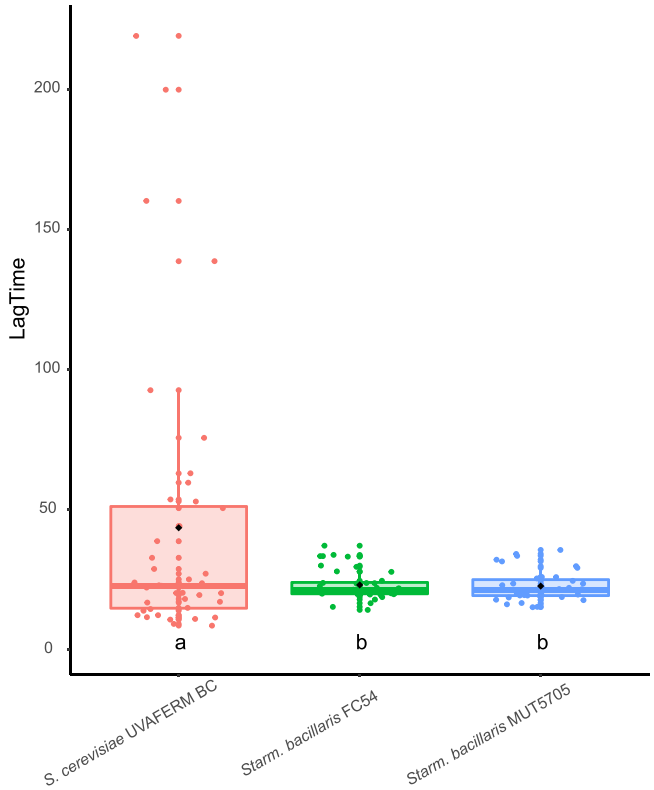

FIG 4 Box plots display the distribution of all of the nitrogen sources for each strain according to their performance in lag phase during fermentation. Lag time is presented in hours.

FC54 and MUT5705), while a substantial fermentation activity was found on His (FC54, 0.20 g/liter/h; MUT5705, 0.26 g/liter/h) and Gln (FC54, 0.17 g/liter/h; MUT5705, 0.23 g/liter/h). Concerning the performance of both strains, *S. bacillaris* MUT5705 displayed in general a better fermentative capacity during the second stage of fermentation than FC54. In particular, $R_{max2stage}$ values measured during FC54 fermentation on Thr, Lys, and, to a lesser extent, on Ala, Gly, and Leu were markedly lower than those of MUT5705 (data not

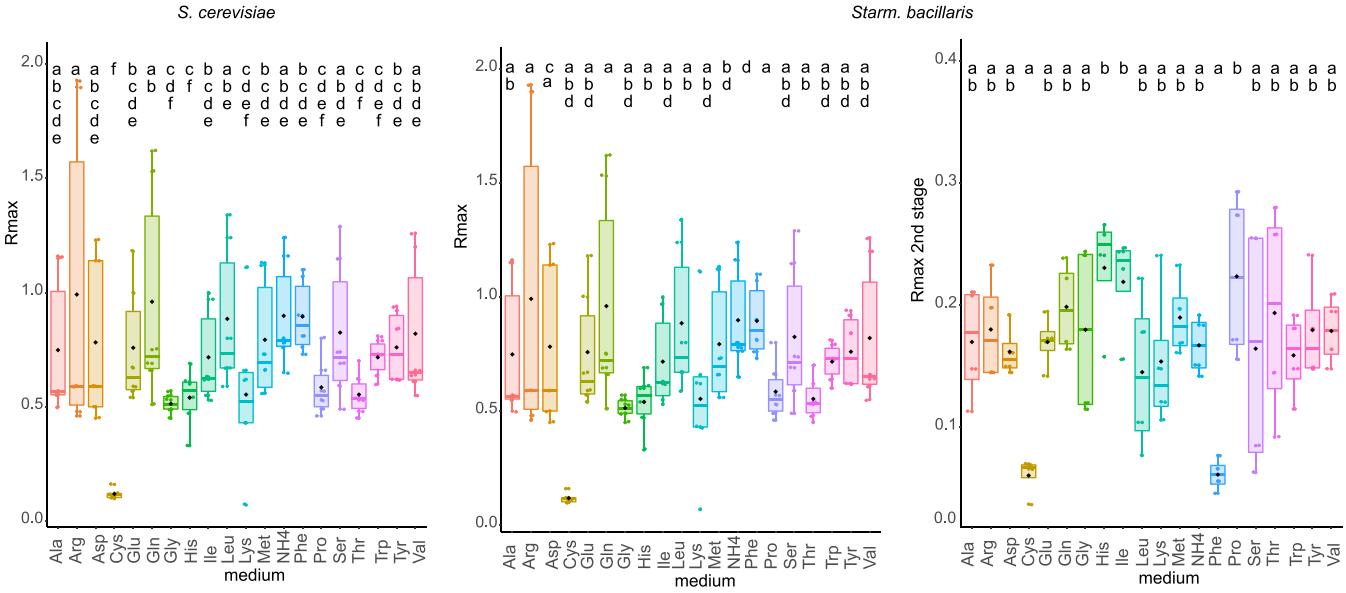

FIG 5 Box plots demonstrate the effect of each nitrogen source on the fermentation rate (R_{max}) registered during the 1st and 2nd phases of fermentation for each strain.

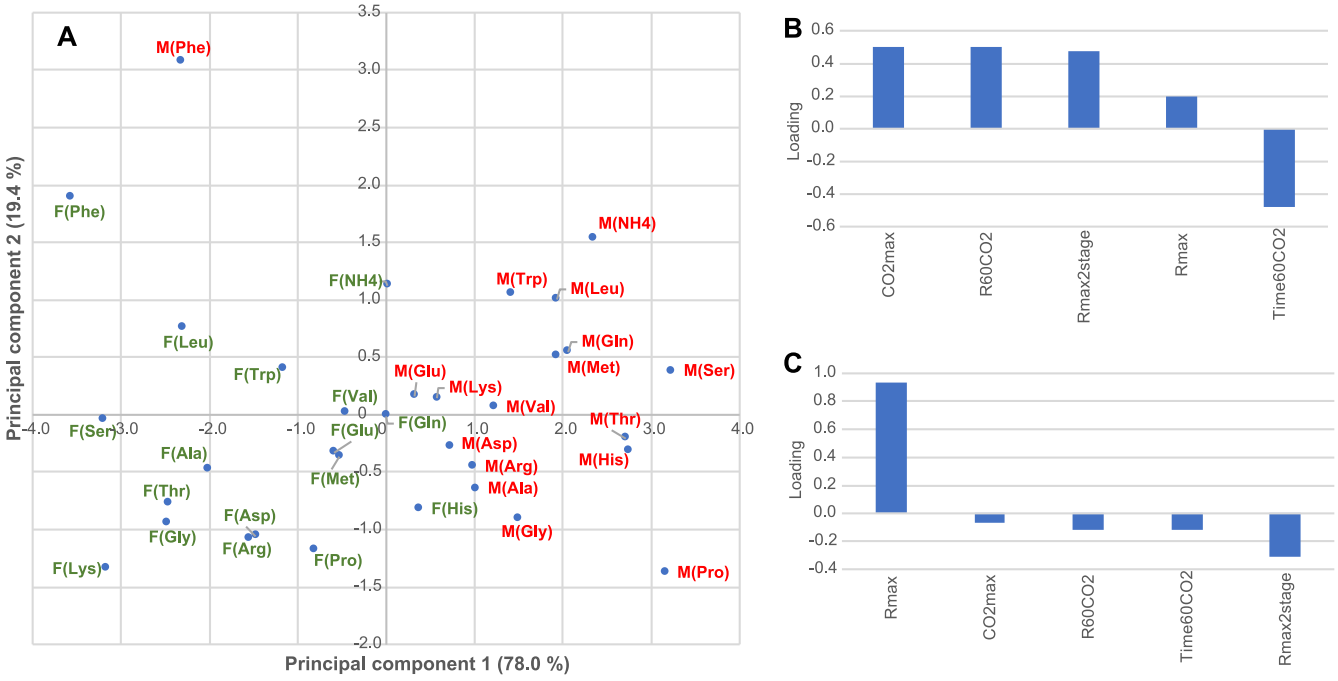

FIG 6 Score plot (A) and loading plots of the first (B) and second (C) principal components corresponding to PCA of the fermentation indexes obtained with the two *Starmerella bacillaris* strains FC54 and MUT5705 in the 18 synthetic media (score plot [A] and loading plot [B]). Points are colored depending on the strain. *S. bacillaris* FC54, green; *S. bacillaris* MUT5705, red. Cystine was removed from the PCA due to the inability of both species to ferment this amino acid.

shown). Furthermore, a fermentation rate of 0.255 g/liter/h was measured during the 2nd part of MUT5705 fermentation with Ser as nitrogen source, while FC54 was still almost unable to ferment after 150 h under these conditions.

Then, in order to differentiate the nitrogen sources according to their efficiency in sustaining fermentation, we first compared the time required to produce 60 g/liter CO_2 (T_{60g}), this parameter taking into account the fermentative activity during the two phases. Overall, strain MUT5705 displayed better fermentative capacities regardless of the nitrogen source, with T_{60g} ranging from 119 to 209 h, lower than those of FC54 (comprised of values between 160 and 283 h). Differences were found in the efficiency of nitrogen sources to support fermentation depending on the strains. While both strains displayed short T_{60g} values during fermentation on NH_4^+, Gln, and Tyr and long T_{60g} values on Phe and Gly, Ser efficiently supported MUT5705 fermentation but poorly supported FC54 fermentation. Conversely, Glu was an efficient nitrogen source to sustain FC54 fermentation but not MUT5705 fermentation. Finally, during fermentation using Thr and His, FC54 displayed long and a short T_{60g} values, respectively, while an effective MUT5705 fermentation was observed on Leu but not on Lys.

Finally, a principal-component analysis (PCA) was performed to provide an overview of the combined incidences of the strain and nitrogen source on *S. bacillaris* fermentation performances (Fig. 6). The two first principal components explained about 97.4% of the total variance. The variables with a positive contribution to the first component (PC1, 78.0% of the variance) were $CO_{2\ max}$, R_{60CO2}, and $R_{max2stage}$, while T_{60g} negatively contributed to the differentiation of the individuals through this axis (coefficients $>$ 0.4). This component permitted a good separation of the 2 *S. bacillaris* strains according to their capacity to maintain substantial fermentation activity during the final part of the process and to complete fermentation, with the majority of *S. bacillaris* MUT5705 individuals grouped on the right part of the graph (except Phe) while FC54 individuals clustered on the left part (except His), reflecting the lower fermentation capacity of this strain. The second principal component (PC2, 19.8% of variance) was mainly positively correlated with R_{max} with a $>$0.8 coefficient and negatively correlated with $R_{max2stage}$. This axis allowed the

differentiation of nitrogen sources according to their efficiency in supporting fermentation. Interestingly, both *S. bacillaris* strains responded similarly for most of the nitrogen compounds used in this study. Among them, NH_4^+, Phe, and Trp allowed both strains to achieve the highest levels of R_{max} while the lowest levels were observed with Pro.

DISCUSSION

The availability and composition of the nitrogen resource are essential factors that govern interactions between yeast species in the wine environment (24, 25). Thus, to fully exploit the properties of interest of the two species used in co- or sequential fermentation, a thorough knowledge of how these yeasts assimilate the nitrogen compounds of grape juice is an important prerequisite (3, 14, 26–28). In the present study, we investigated the efficiency of 18 nitrogen sources for sustaining the growth and fermentation of two *S. bacillaris* strains in comparison with an *S. cerevisiae* strain. Nitrogen was provided as 200 mg/liter for each of the 18 individual nitrogen sources (17 amino acids and ammonium) achieving a YAN concentration above the recommended minimum level for *S. cerevisiae* for a complete fermentation at a normal rate (29). Based on divergent behaviors and performances of the two species, some phenotypic characteristics of the *S. bacillaris* strains were evidenced.

Regarding growth, it is important to consider the differences between *S. bacillaris* and *S. cerevisiae* strains with respect to the metabolic pattern involved in the assimilation of each amino acid and, consequently, to the genetic specificities of both species (30). First, contrary to *S. cerevisiae*, both *S. bacillaris* strains were able to grow in media supplemented with Lys, His, and Cys. Interestingly, the first step of the His and Lys assimilation pathway, which allows nitrogen uptake, is not catalyzed by the general α-transaminase (encoded by *BAT1* and *BAT2* in *S. cerevisiae*) but uses different metabolic routes. Five pathways have been described in yeasts and fungi for Lys degradation (31), involving either a lysine N^6-acetyltransferase, a lysine racemase, a lysine 6-dehydrogenase, an ε-aminotransferase lysine, or a pyruvate 6-aminotransferase. These pathways have never been identified in *S. cerevisiae* isolates, confirming the inability of this yeast to consume Lys as sole nitrogen source (32). However, they were detected in other yeast species, including some species close to the *Starmerella* clade (33, 34), which is in line with the results of the present study. The main metabolic route responsible for His assimilation in most organisms involves, in a first step, a histidine ammonia-lyase. However, the gene coding for this enzyme has been lost in some yeasts, including *S. cerevisiae* and *Candida glabrata* (35). This latter species initializes His degradation via the Aro8p aminotransferase, whose activity and stability have been adapted throughout evolution for the effective use of histidine. In *S. cerevisiae*, the transsulfuration pathway is able to convert Cys and homocysteine to one or the other side. Cys degradation involved the combination of this compound with *O*-acetylhomoserine to form cystathionine, catalyzed by cystathionine-synthase, further cleaved by cystathionine-lyase, yielding pyruvate, ammonium, and homocysteine. In *S. cerevisiae*, cystathionine-synthase and -lyase are encoded by *STR2* and *STR3*, respectively (36). On the basis of these considerations, it may be assumed that the high capacity of *S. bacillaris* strains to grow in the presence of His, Lys, and Cys is likely related to either the presence and expression or the evolutionary fitness of specific genes responsible for the catabolism of these amino acids. Further comparative genomics analyses would help elucidate the differences observed between the two species.

Quite surprisingly, *S. bacillaris* strains displayed a higher capacity than *S. cerevisiae* to assimilate glycine and amino acids associated with the Ehrlich pathway, *viz.*, Leu, Met, Phe, Thr, Trp, and Val (37), with usually an increased growth rate and a shorter lag phase. This pathway involves an initial transamination of amino acids to branched-chain 2-oxo acids, further decarboxylated to aldehydes (37). First, the presence of more efficient transaminases (mainly Bat1p, Bat2p, Aro9p, and Aro8p) or a higher expression of transaminase-coding genes in *S. bacillaris* strains compared to *S. cerevisiae* have to be considered to explain growth differences when Ehrlich amino acids were used as

sole nitrogen sources. Interestingly, a recent study (38) highlighted the lack of a *PDC1* orthologue in the *Wickerhamiella*/*Starmerella* clade, which is the gene coding for the main pyruvate decarboxylase in *S. cerevisiae* (39). To compensate for this loss, the role of pyruvate decarboxylase was taken over in *Starmerella* strains by a distinct enzyme, encoded by the *ARO10* gene (40) with a broader substrate specificity than Pdc1p (41, 42). In addition, to explain the redirection of fluxes in the central carbon metabolism (CCM) of *S. bacillaris* as previously reported (26), these genomic features may contribute to the higher capacity of *S. bacillaris* strains to use branched and aromatic amino acids as sole nitrogen sources compared to *S. cerevisiae*.

Conversely, *S. cerevisiae* grew better than *S. bacillaris* on NH_4^+, Arg, and Ser as sole nitrogen sources, likely as a result of variations in the coding sequences or in the regulatory regions of genes involved in the assimilation of these amino acids. Finally, when amino acids directly related to the central nitrogen core, *viz.*, Asp, Glu, and Gln, were provided as sole nitrogen source, the growth profile of the two species was very similar (less than 20% variation), suggesting that this key part of the microbial metabolism is strongly conserved between yeast species. Overall, differences in the allocation of nitrogen compounds as preferred, intermediate, and nonpreferred sources to support *S. bacillaris* growth were found with *S. cerevisiae* and, more generally, with other yeast species (3). Interestingly, Leu was considered a "preferred" amino acid for *S. bacillaris* and *Metschnikowia pulcherrima* but "intermediate" for *S. cerevisiae*; similarly, *S. bacillaris*, *M. pulcherrima*, and *Torulaspora delbrueckii* were able to grow in the presence of Lys, which *S. cerevisiae* is unable to assimilate. Conversely, Ser efficiently supported growth of *S. cerevisiae* and *M. pulcherrima* but not of *S. bacillaris*.

In a previous study (26), using a synthetic medium and a mixture of nutrients, the poor fermentation activity of *S. bacillaris* compared to *S. cerevisiae* has been demonstrated. Consistent with this, while both *S. bacillaris* strains exhibited a better growth capacity than *S. cerevisiae* on the vast majority of single nitrogen sources, their fermentation performances were inferior to those registered for *S. cerevisiae* for all amino acids. The only exceptions to this general pattern concerned the *S. bacillaris* strain MUT5705 that was able to achieve fermentation to dryness when nitrogen was supplied as Ser, Thr, Pro, or His. This discrepancy between growth and fermentation performances may be explained by the fact that successful completion of the fermentation process depends not only on a satisfactory cell growth but also on yeast ability to tolerate relatively high levels of ethanol and to efficiently consume both fructose and glucose (19).

Although *S. bacillaris* strains tolerate relatively high levels of ethanol (43), their resistance to this stress is nevertheless lower than that of *S. cerevisiae* (44, 45), which might slow down their activity during the last stage of fermentation.

In fermentations performed with *S. bacillaris*, a break in the evolution of the fermentation rate over time occurred after approximately 135 h to 150 h of culture, which is not observed during *S. cerevisiae* fermentations. This observation could be explained by the limited capacity of the former species to use glucose as a carbon source (26). Thus, unlike *S. cerevisiae*, which favors glucose consumption, *S. bacillaris* consumes fructose preferentially to glucose and, therefore, is considered a fructophilic yeast (46, 47). The *S. bacillaris* strain FC54 displayed the most important change in fermentative activity, highlighting the lower capacity of this strain to consume glucose compared with *S. bacillaris* MT5707. This specific feature of *S. bacillaris* strains contributes to a better understanding of the differences between the efficiency of nitrogen sources for sustaining growth on one hand and for fermentation support on the other hand. The change in the fermentation rate after fructose exhaustion is more or less pronounced depending on the amino acid and the strain, markedly more in the presence of Thr, Phe, and Ser than Glu, Gln, and Asp. These findings led us to hypothesize the presence of different regulation mechanisms and underlines the need for further investigation.

It is worth noticing the shorter lag phase of *S. bacillaris* strains in the presence of some amino acids (Leu, Pro, Trp, and Val) compared to that of *S. cerevisiae*. The addition of these nitrogen sources could help *S. bacillaris* to establish itself in the grape must and dominate over the indigenous microbiota by increasing its cell number prior to *S. cerevisiae* inoculation. This consideration may be particularly relevant for winemaking, as the increase in the metabolic activity of the inoculated yeast could impart a positive impact on the wine's chemical and sensory quality (19)

Recently, marked differences were demonstrated between *S. bacillaris* and *S. cerevisiae* in the sequence of nitrogen compound uptake when provided as a mixture of ammonium and amino acids (26). Taking into consideration the results of the current study, no correlations were found between the order of consumption of nitrogen compounds reported in this work and their capacity to support growth and fermentation in *S. bacillaris* strains. The most evident feature concerned ammonium, preferentially and entirely consumed by *S. bacillaris* strains at the expense of amino acid assimilation. However, NH_4^+ was the less efficient nitrogen source for growth support when used as the unique nitrogen source. Conversely, Asp, Leu, and His efficiently supported *S. bacillaris* growth and fermentation but were not taken up during fermentation with a mixture of nitrogen sources. This reflects that different mechanisms control, on one hand, the transport of nitrogen sources and thus their sequence of entry into the cells and, on the other hand, amino acid catabolism and consequently their efficiency to sustain fermentation. Overall, these observations highlight the complexity of nitrogen management to control sequential fermentations with *S. bacillaris* and *S. cerevisiae* and underline the necessity to further investigate the different mechanisms involved.

In conclusion, this work offers a comprehensive picture of *S. bacillaris* preferences with respect to nitrogen sources for sustaining growth and fermentation, with important differences compared to the reference yeast *S. cerevisiae* regarding rates, final population, and CO_2 production. Interestingly, no correlation was shown between the ability of nitrogen sources to support growth and their fermentation efficiency. This phenotypic diversity can certainly be explained by different genetic backgrounds, but further investigations are needed to elucidate the molecular mechanisms involved. Furthermore, only one *S. cerevisiae* strain and two *S. bacillaris* strains were compared in this study, and a characterization of a larger collection of *S. bacillaris* strains is required to assess the genericity of the nitrogen source preferences in this species, as the efficiency of nitrogen sources to support growth and fermentation could be strain dependent. The data from the current study highlight the importance of appropriate management of the nitrogen resource during co- or sequential fermentation combining *S. bacillaris* and *S. cerevisiae*. Such improved understanding could help winemakers to set up a nitrogen nutrition scheme adapted to the requirement of each species during fermentation by supplementing with relevant nitrogen compounds. This will prevent nitrogen depletion or competition between yeasts for nitrogen sources and, consequently, possible issues to complete fermentation.

MATERIALS AND METHODS

Strains. *S. cerevisiae* (Uvaferm BC; Lallemand Inc., Montreal, Canada) and two *S. bacillaris* strains, FC54 and MUT5705 (Department of Agricultural, Forest and Food Sciences [DISAFA], University of Torino, Italy), were used. All strains were maintained at 4°C as slants in yeast extract-peptone-dextrose (YPD) agar plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar; all from Oxoid, Paris, France).

Inoculation procedure. Initial cultures were performed by inoculating three independent colonies for each strain (representing three biological replicates) in 5 ml YPD medium and incubated at 28°C for 24 h. Then the preinocula were subcultured in 70 ml YPD medium in 100-ml Erlenmeyer flasks and incubated with shaking (150 rpm) at 28°C for 24 h. Subsequently, these cells were transferred to 250-ml Erlenmeyer flasks containing 150 ml YNB (0.67% yeast nitrogen base with amino acids and ammonium from Difco, Detroit, USA, supplemented with 1% fructose and 1% glucose) previously sterilized by filtration with a 0.2-μm membrane filter (VWR, Paris, France) at an initial cell population of 1.0×10^6 cells/ml and further incubated with shaking (150 rpm) at 28°C for 24 h. After this period, an aliquot of cells was centrifuged at 3,000 rpm at 25°C for 5 min, and the resulting pellet was washed twice with a sterile 8 g/liter NaCl physiological solution. Cells were thereafter resuspended in 100 ml of YNB without amino acids or ammonium (but supplemented with 1% fructose and 1% glucose) to obtain a concentration of 1.0×10^8 cells/ml and incubated under the above-mentioned conditions for 2 h to eliminate all intracellular nitrogen reserves.

Cell density. Total cell density was determined using a Beckman Coulter counter (Model Z2; Margency, France), fitted with a 100-μm aperture probe, after sample dilution with Isoton II (Beckman-Coulter, Margency, France).

Fermentation medium. Phenotypic parameters were monitored using a synthetic medium (MS200) at pH 3.3 that mimics a natural grape must (29) with the following modifications: 100 g/liter fructose, 100 g/liter glucose, and without amino acids or ammonium. Ergosterol (1.875 mg/liter), oleic acid (0.625 mg/liter), and Tween 80 (0.05 g/liter) were provided as anaerobic factors. To test the ability of each strain to grow on and ferment different N sources, 19 fermentations were performed as follows: 17 fermentations with a single amino acid (at 200 mg N/liter), 1 fermentation with ammonium sulfate (200 mg N/liter), and 1 fermentation without N source as negative control. The 19 amino acids used were alanine (Ala), arginine (Arg), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophane (Trp), and valine (Val) (all from Sigma, Paris, France). Each "must" was flash-pasteurized prior to growth and fermentation assays.

Growth phenotypic assays. A total of 198 μl of synthetic medium prepared as described above was added to the 96 wells of flat-bottomed microplates. Subsequently, 2 μl of precultures corresponding to an initial cell density of 1.0×10^6 cells/ml was introduced to each well for a final volume of 200 μl (initial optical density at 600 nm [OD_{600}] $\approx$ 0.005 to 0.02) in biological triplicates. Some wells were not inoculated for background correction purposes. Microplates were then shaken in a thermostated microplate reader (Tecan Infinite M200 Pro) at 25°C for 154 cycles of orbital (480 s at 3 mm) and linear (120 s at 3 mm) shaking in order to resuspend the cells before OD measurements (about 48 h in total). Using the microplate reader software, raw OD data were exported as Excel tables with the addition of kinetic time stamps and temperature values. Growth was determined by comparing the optical density of each strain with and without the presence of a specific nitrogen compound. Meanwhile, the growth parameters—lag phase time (h), maximum growth rate (μ_{max} [1/h]), and maximum OD (OD_{max})—were calculated using the equations described by Su et al. (3).

Fermentation assays. Fermentations were performed in 15-ml glass fermenters, equipped with fermentation air locks, containing 10 ml of synthetic medium, and inoculated with the abovementioned inoculum at an inoculation density of 1.0×10^6 cells/ml. Fermentations were carried out for 14 days under isothermal conditions (25°C) with continuous magnetic stirring (300 rpm). Fermentation was monitored by automatically measuring every 20 min the fermenting medium weight loss due to CO_2 emission. CO_2 production rate was calculated by polynomial smoothing of the last 10 weight loss measurements (48). The frequent acquisitions of weight loss and the precision of weighing allowed the highly accurate calculation of the CO_2 production rate. The following fermentation parameters were calculated: maximum CO_2 production (g/liter), maximal fermentation rate (R_{max}) (g/liter/h), time to achieve R_{max} (h), CO_2 production at R_{max} (g/liter), lag phase time (h), time to achieve 60 g CO_2 production (T_{60g}) (h), fermentation rate measured after production of 60 g CO_2 (R_{60CO2}) (g/liter/h), and $R_{max2stage}$ (g/liter/h). The 2nd phase observed in *S. bacillaris* fermentation profiles corresponded to a substantial decrease in the fermentation rate due to fructose exhaustion.

Nitrogen determination. Initial amino acid concentrations in the musts were determined by liquid chromatography using an amino acid analyzer (Biochrom 30; Biochrom Ltd., Cambridge, UK). Amino acids were separated using an ion-exchange column (Ultrapac-8 Lithium form; Amersham Pharmacia Biotech) and detected by reaction with ninhydrin using the chromatographic conditions reported by Crépin et al. (14). The quantification of each individual amino acid was calculated based on peak areas relative to a norleucine (Sigma, Paris, France) internal standard, with a reference to an external standard mixture of amino acids. The ammonium concentration in synthetic must supplemented with ammonium sulfate only was determined enzymatically with an enzymatic assay ammonia assay kit (R-Biopharm AG, Darmstadt, Germany) as described by the manufacturer.

Statistical analyses. Significant differences among strains were established using a one-way analysis of variance (ANOVA) test, employing Tukey's honestly significant difference (HSD) *post hoc* test at a significance level of $P < 0.05$. Heat maps were based on agglomerative hierarchical clustering of the growth parameters with the Euclidean distance similarity metric and average linkage method. Statistical analyses were carried out using IBM Statistics software package (SPSS, Chicago, IL, USA).

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