Specific phenotypic traits of Starmerella bacillaris regarding nitrogen source consumption and central carbon metabolites production during wine fermentation

Specific phenotypic traits of *Starmerella bacillaris* regarding nitrogen source consumption and central carbon metabolites production during wine fermentation

Vasileios Englezos[1, 3], Luca Cocolin[1], Kalliopi Rantsiou[1], Anne Ortiz-Julien[2], Audrey Bloem[3], Sylvie Dequin[3], Carole Camarasa[3*]

[1]University of Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Agricultural Microbiology and Food Technology Sector, Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy.

[2]Lallemand SAS, Blagnac, France.

[3]SPO, INRA, SupAgro, Université de Montpellier, F-34060 Montpellier, France

[*]Corresponding author: Carole Camarasa, email: camarasa@supagro.inra.fr.

ABSTRACT

Over the last past years, the potential of non-Saccharomyces yeasts to improve the sensory quality of wine has been well recognized. In particular, the use of *Starmerella bacillaris* in mixed fermentations with *Saccharomyces cerevisiae* was reported as an appropriate way to enhance glycerol formation and reduce ethanol production. However, during sequential fermentation, many factors as the inoculation timing, strain combination and physical and biochemical interactions can affect yeast growth, fermentation process and/or metabolite synthesis. Among them, yeast assimilable nitrogen (YAN) availability, due to its role in the control of growth and fermentation, has been identified as a key parameter. Consequently, a comprehensive understanding of the metabolic specificities and the nitrogen requirements would be valuable to better exploit the potential of *Starm. bacillaris* during wine fermentation. In this study, marked differences in the consumption of the total and individual nitrogen sources were registered between the two species, while the two *Starm. bacillaris* strains generally behaved uniformly. *Starm. bacillaris* strains are differentiated by their preferential uptake of ammonium compared with amino acids that are poorly assimilated or even produced (alanine). Otherwise, the non-*Saccharomyces* yeast exhibits low activity through the acetaldehyde pathway, which triggers an important redistribution of fluxes through the central carbon metabolic network. In particular, the formation of metabolites deriving from the two glycolytic intermediates glyceraldehyde-3-phosphate and pyruvate is substantially increased during fermentations by *Starm. bacillaris*. This knowledge will be useful to better control the fermentation process in mixed fermentation with *Starm. bacillaris* and *S. cerevisiae*.

IMPORTANCE

Mixed fermentations using a controlled inoculation of *Starm. bacillaris* and *S. cerevisiae* starter cultures represent a feasible way to modulate wine composition that takes advantage of both the phenotypic specificities of the non-*Saccharomyces* strain and the ability of *S. cerevisiae* to complete wine fermentation. However, according to the composition of grape juices, the consumption by *Starm. bacillaris* of nutrients, in particular of nitrogen sources, during the first stages of the process may result in depletions that further limit the growth of *S. cerevisiae* and lead to stuck or sluggish fermentations. Consequently, understanding the preferences of non-*Saccharomyces* yeasts for the nitrogen sources available in grape must together with their phenotypic specificities is essential for an efficient implementation of sequential wine

fermentations with *Starm. bacillaris* and *S. cerevisiae* species. The results of our studies demonstrate a clear preference for ammonium compared to amino acids for the non-*Saccharomyces* species. This finding underlines the importance of nitrogen sources, which modulate the functional characteristics of inoculated yeast strains to better control the fermentation process and product quality.

Introduction

Spontaneous wine fermentation is a complex process that is carried out by a succession of different yeast species and strains within a species that are resident populations of the winery or vineyard where grapes are grown (1). This fermentation practice allows wines to express the complexity of the vineyard microbiota and allows wine consumers to experience the nuances between different vineyards and vintages (2). The high degree of complexity that characterizes these wines is derived from an array of by-products produced from different native non-*Saccharomyces* and *Saccharomyces cerevisiae* yeasts (3). However, the evolution of agronomical practices together with climate variations increasing the average mean temperature in many wine regions has resulted in higher sugar contents in grapes and, consequently, in musts (4). Not to mention that some wild yeasts can play a negative role in the character of the product, through the production of undesirable metabolites, such as acetaldehyde, hydrogen sulphide and volatile acidity. Furthermore, spontaneous fermentations lacks reproducibility (1). As a consequence, producers are often forced to inoculate with selected yeasts to avoid stuck fermentations and/or produce wines with a wide range of complex flavors and aromas (2). Therefore, many winemakers inoculate musts with commercial *S. cerevisiae* strains to ensure a rapid increase in the *S. cerevisiae* cell number, to improve the fermentation rate and to produce more predictable wines with established criteria (5).

Along with the addition of a *S. cerevisiae* strain, the use of mixed starter cultures with selected non-*Saccharomyces* and *S. cerevisiae* yeasts by simulating spontaneous fermentation can result in a greater complexity of wine and produce unusual aromas and flavors in ways not that cannot be attained with a pure starter culture of *S. cerevisiae* (6). Production of these complex aromas and flavors is mainly due to the ability of the non-conventional species to produce target metabolites or hydrolyze aromatic precursors (7). Despite these positive aspects, in recent years, a concern regarding the use of sequential mixed culture fermentations has been

noted because the initial growth of non-*Saccharomyces* yeasts may compete with *S. cerevisiae* for nutrients, limiting their subsequent growth and increasing the risk of sluggish or stuck fermentation (8).

The lacking of nitrogen, in the form of ammonium and amino acids (YAN), is often involved in problematic fermentation. This resource plays an important role in the fermentation progress since it is essential for the growth and metabolic activity of yeasts. The nitrogen compounds are rapidly consumed by yeast cells during the first 24-36 h of fermentation to fill the biosynthetic pools of amino acids necessary for protein synthesis and growth (9). Moreover, the ability of strains to complete fermentation depends on the level of biomass production (10-11), while nitrogen deficiency results in a lower biomass yield, which in turn, decreases the fermentation rate and increases the time to complete fermentation. The absolute minimum concentration of nitrogen required for the completion of fermentation is very difficult to determine since the temperature, initial sugar concentration and genetic background of the strain all modulate this parameter (12-13). It is also important to note that not all nitrogen sources equally support yeast growth because cells growing on ammonium, asparagine or glutamine as the sole nitrogen source exhibit a 2 h-generation time, while the generation time is increased by up to 4.5 h when yeasts are grown on tryptophan (14). Moreover, in the presence of amino acids and ammonium, wine yeasts sequentially uptake nitrogen sources, and the order of assimilation is controlled by molecular mechanisms (15).

Among non-*Saccharomyces* yeasts, *Starmerella bacillaris* can occur at high numbers in grape musts (16). This species is known for its strong fructophilic character and its ability to produce low ethanol and high glycerol concentrations (17). Taking into consideration these characteristics, the coupling of *Starm. bacillaris* with selected *S. cerevisiae* strains has been proposed to improve wine. In particular, sequential fermentation with *Starm. bacillaris* and *S. cerevisiae* strains results in the reduction of ethanol in wines, which is a current challenge in the context of the constant increase in the sugar content of grape juice due to global climate change (18, 19). However, the achievement of fermentation and the final metabolite profiles are strain-dependent and depend on having a fermentation environment, especially in regard to the delay between the *Starm. bacillaris* and *S. cerevisiae* inoculations (18,20,21). One of the most probable explanation for these observations that is worthwhile to investigate is a more pronounced exhaustion of nitrogen sources by *Starm. bacillaris* when *S. cerevisiae* is added, resulting in the limited implantation of this species.

In light of this evidence, a comprehensive exploration of the assimilation of complex nitrogen sources by both partners would be valuable to better exploit the potential of *Starm.*

bacillaris during sequential fermentation with *S. cerevisiae*. To this end, the aim of this study was to evaluate nitrogen assimilation from complex nitrogen compounds (amino acids and ammonium) by *Starm. bacillaris* and *S. cerevisiae* during pure culture fermentations, as well as to investigate the sequence of assimilation. The chemical composition of wines were compared to each other to evaluate the impact of each species on the final product.

MATERIALS AND METHODS

Yeast strains. Two *Starm. bacillaris* strains and one *S. cerevisiae* strain were used in this study. The *Starm. bacillaris* strains were FC54 and MUT705 from the yeast culture collection of DISAFA (Department of Agricultural, Forest and Food Sciences, University of Torino, Italy) and MUT (Mycotheca Universitatis Taurinensis, DBIOS, University of Torino, Italy), respectively. The commercial *S. cerevisiae* strain Uvaferm BC® (Lallemand, Inc., Montreal, Canada) was used as a reference strain.

Inoculation procedure. For each strain, an aliquot of frozen cells (maintained at – 80 °C) was propagated at 28 °C in YPD broth (1% yeast extract, 2% peptone and 2% glucose, all from Oxoid, Paris, France) and streaked onto YPD agar plates to obtain single colonies 72 h before fermentation. Afterwards, one fresh colony was selected to inoculate 10 mL of YPD medium in a 50 mL Erlenmeyer flask at 28 °C with continuous shaking (150 rpm). After 24 hours of incubation, an aliquot of culture was used to inoculate 10 mL of synthetic or natural grape must at an initial cell population of 1.0 x 10^6 cells/mL. The inoculum was grown under the same conditions for another 24 hours.

Fermentation media. Fermentations were performed in synthetic medium, called SM200, which simulates standard grape juice at pH 3.3. The medium was prepared using the protocols described by Bely et al. (22) with the following modifications: 114.7 g/L glucose, 114.5 g/L fructose, and 202 ± 5.4 mg/L yeast assimilable nitrogen (YAN) as a mixture of 19 amminoacids (132.9 ± 3.9 mg N/L) and ammonium salt (69.1 ± 1.5 mg N/L). Fermentations were performed in duplicate in 1.2 L glass fermenters containing 1.1 L of synthetic medium that was previously flash-pasteurized and inoculated with 1.0 x 10^6 cells/mL using the abovementioned inoculum. Fermenters were equipped with fermentation air-locks to maintain semi-anaerobic conditions and incubated at 25 °C with continuous magnetic stirring (300 rpm). Fermentations were stopped when the weight loss remained constant for two consecutive days. The reference medium (SM200) was supplied with various mixtures of amino acids and ammonium to form 3 different musts (Table 1). The composition of the musts was as follows

(in mg N/L): SMA (200.3 ammonium), SM200B (177.3 amino acids and 22.9 ammonium) and SM200C (206.1 amino acids). These fermentations were conducted in duplicate in 330 mL glass fermenters using the abovementioned fermentation conditions.

Analytical methods. Cell densities were monitored every 3 hours from 12 to 48 hours and then once a day from 48 to 96 hours of fermentation by counting cells using an electronic particle counter (Multisizer 3 Coulter Counter, Beckman Coulter) after sonication to separate aggregated cells. Cell viability during the middle-end phases of fermentation was determined with an epifluorescent method using a C6 cytometer (Accuri, BD Biosciences, San Jose, CA) as described by Delobel et al. (23). Briefly, cells were stained with propidium iodide (PI), an indicator of cell viability that works due its inability to penetrate intact cell membranes. Viability was determined as the percentage of intact and fragile cells among all cells. Each sample was analyzed using three biological replicates.

The total YAN concentration was determined according to the sum of organic (amino acids) and inorganic nitrogen (ammonium). Before the quantification of free amino acids, molecules with high molecular weights were removed from the samples by the addition of 200 µL of a sulfosalicylic acid solution (25 w/v) to 800 µL of sample, followed by incubation at 4 °C for 1 h. After centrifugation at 14000 rpm for 10 min, the samples were filtered through a 0.22 µm pore-size Millipore nitrocellulose membrane. Amino acid identification and quantification were performed by liquid chromatography with a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK) using the chromatographic conditions reported by Crepin et al. (15). The ammonium concentration was assayed spectrophotometrically using an enzymatic kit (R-Biopharm AG™, Darmstadt, Germany) according to the manufacturer's instructions.

The extracellular sugar, ethanol, glycerol and organic acid (acetic, fumaric, pyruvic, a-ketoglutaric and succinic acid) concentrations in the samples were determined by HPLC (HPLC 1290 Infinity, Agilent Technologies, Santa Clara, California, USA) using an HPX-87H ion exclusion column (Bio-Rad). The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min. The organic acid concentrations were determined with a UV meter at 210 m, while the concentration of the other compounds was determined with a refractive index detector (24). A total of 23 volatile metabolites were identified in the fermented wines, and these compounds included 5 higher alcohols, 4 acetate esters, 7 ethyl esters and 7 volatile acids. Analyses were performed by gas chromatography-mass spectrometry according to the protocols reported by Rollero et al. (25). The accuracy of the quantification of the metabolites was achieved with the use of poly-deuterated internal standards for stable isotope dilution analysis (26).

Statistical analyses. Differences were established using one-way Analysis of Variance (ANOVA), followed by the software IBM SPSS Statistics package (version 19.0, IBM Corp., Armonk, NY, USA). ANOVA analysis was coupled with the Tukey-b post hoc test when *p* values were lower than 0.05 to evaluate significant differences.

RESULTS

Growth and metabolite evolution during fermentation. *Starm. bacillaris* and *S. cerevisiae* strains were grown in duplicate in SM200 synthetic medium with a high sugar concentration (229 g/L) and 202 mg/L of YAN, which consisted of a mixture of 19 amino acids and ammonium ions. The growth and the kinetics of metabolite formation from central carbon metabolism (CCM) were monitored according to the fermentation and profiles of the produced volatile compounds determined at the end of culturing.

Both the growth and metabolite dynamics differed considerably between the two species, while the two *Starm. bacillaris* strains generally behaved uniformly (Fig. 1, Table 2). *S. cerevisiae* Uvaferm BC® reached a maximum population of 1.0 x 10^8 cells/mL in 36 hours and simultaneously consumed glucose and fructose, with a preference for glucose (118 vs. 142 hours for exhaustion). By contrast, a completely different picture emerged when *Starm. bacillaris* strains were used to ferment the must. Fermentation proceeded more slowly compared to *S. cerevisiae* and stopped after 340 hours. At this stage, almost all of the available fructose had been consumed (residual fructose: 3.7 – 11.3 g/L), while glucose remained untouched (residual glucose: 106.5 – 107.1 g/L). Furthermore, both strains exhibited a similar growth dynamics pattern, reaching a cell population of about 7.6 x 10^7 cells/mL in 48 hours.

The *Starm. bacillaris* strains were clearly differentiated from *S. cerevisiae* as they produced high amounts of glycerol and organic acids and low amounts of ethanol and acetic acids (Table 2). Glycerol production was very similar for both yeast species (7.7 to 8.2 g/L) despite the differences in their sugar consumption levels. This similarity was due to the higher glycerol yields of *Starm. bacillaris* strains (69.7 – 76.5 mg/g) compared to that of *S. cerevisiae* (50 mg/g). Ethanol was significantly increased in wines fermented with *S. cerevisiae*, in accordance with the higher sugar consumption of this species. However, *Starm. bacillaris* strains displayed lower ethanol yields (a reduction of 2.7 mg/g) compared to Uvaferm BC® (Table 2).

Large differences between *S. cerevisiae* and *Starm. bacillaris* strains were also found in regard to the yields of organic acids. First, the acetic acid yield of *Starm. bacillaris* strains (1.5 and 1.8 mg/g) was more than two times lower than that of *S. cerevisiae* (3.9 mg/g).

Combined with the inefficient consumption of sugars by *Starm. bacillaris*, the reduced yield of acetic acid resulted in an important decrease in the formation of this compound during *Starm. bacillaris* fermentation (0.11-0.21 g/L instead to 0.64 g/L for *S. cerevisiae*). A similar pattern was observed in the production of succinic acid, with a lower production in *Starm. bacillaris* strains (0.13-0.24 g/L) than in *S. cerevisiae* (0.80 g/L) (Table 2). Conversely, the non-*Saccharomyces* strains exhibited higher yields of fumaric, pyruvic and a-ketoglutaric acids than *S. cerevisiae*, resulting in increases of 77%, 77 to 87% and 64% of their final concentration, respectively. A significant decrease in pH with a parallel increase in titratable acidity of 0.67 – 0.94 g/L was seen for wines produced using *Starm. bacillaris* strains. The differences were higher in wines produced from *Starm. bacillaris* MUT 5705.

Higher alcohols were the most predominant volatile metabolite family in the produced wines, followed by acetate esters, ethyl esters and volatile acids (Table 3). Substantial differences were found among the profiles of these aromas in wines produced by *Starm. bacillaris* strains compared to those produced by *S. cerevisiae*. Overall, the final concentrations of volatile metabolites, regardless of their family, were significantly lower in wines produced by *Starm. bacillaris* strains. In particular, the production of acetate and ethyl esters and of all of the volatile acids except butyric acid was strongly reduced in fermentation by *Starm. bacillaris* strains, while sugar consumption was only reduced by half. A 40-, 15- and 7-fold decrease in the formation of acetate esters, ethyl esters and volatile acids by *Starm. bacillaris* was observed compared to those of *S. cerevisiae* Uvaferm BC®, respectively. Conversely, the differences between strains in regard to the production of higher alcohols strongly depended on the nature of each individual compound. First, we found a substantial decrease in the formation of methionol, 2-phenyl-1-ethanol and 3-methyl-1-butanol by *Starm. bacillaris* FC54 and MUT5705, which only accounted for 14-19%, 12-15% and 13-17%, of those produced by *S. cerevisiae* Uvaferm BC®, respectively. On the contrary, the production of propanol by *Starm. bacillaris* strains increased by 1.8-fold compared to that produced by *S. cerevisiae* Uvaferm BC®. In the same way, a pronounced increase in the formation of 2-methyl-propanol was observed, while *S. cerevisiae* Uvaferm BC® produced approximately 74 mg/L2-methyl-propanol, *Starm. bacillaris* FC54 and MUT5707 exhibited a final production of 165 and 148 mg/L2-methyl-propanol, respectively. Finally, *Starm. bacillaris* strains displayed a low ability to synthetize both acetate and ethyl esters compared with *S. cerevisiae* strains, which could be explained by a low efficiency or a lack of acetyl transferases in this species.

Nitrogen consumption. (i) Nitrogen uptake. The profiles of total YAN, amino acids and ammonium consumption by *S. cerevisiae* and *Starm. bacillaris* strains were monitored

during the fermentation process (Fig. 2). Amino acids, alanine, glutamic acid, glycine, leucine, and valine were removed from the graphs due to the ability of *Starm. bacillaris* strains to produce these nitrogen compounds. Proline was also removed since none of the *Starm. bacillaris* and *S. cerevisiae* strains were able to consume this amino acid. All strains mainly consumed YAN during their growth phase: the first 36 h and 48 h of fermentation for *S. cerevisiae* and *Starm. bacillaris*, respectively. However, the pattern of nitrogen consumption differed substantially between the two species. YAN was assimilated faster and at a greater quantity by *S. cerevisiae* Uvaferm BC®. In particular, YAN was entirely exhausted after 30 h of Uvaferm BC® fermentation, while the YAN concentration only decreased to 58 (41%) – 111 (64%) mg N/L when the *Starm. bacillaris* strains reached the stationary phase. At this stage, both amino acids and ammonium remained at considerable amounts, independent of the *Starm. bacillaris* strain. However, ammonium continued to be consumed throughout the stationary phase and was fully depleted after 150 h of culture. On the contrary, *Starm. bacillaris* MUT5705 and FC54 consumed only 50% and 20% of amino acids, respectively. Importantly, 50 – 80% of the available amino acids were still present in the medium at the end of the monitored period.

(ii) The order of amino acid and ammonium uptake. To further investigate the variations between species in regard to their nutritional requirements for nitrogen, the consumption profiles of each N-source during fermentation by the 3 strains were determined (Fig. 3). All of the strains displayed a sequential assimilation of the 20 nitrogen sources provided in the SM200 medium. *S. cerevisiae* Uvaferm BC® was able to exhaust all of the amino acids provided in the synthetic grape juice except proline, according to the order of assimilation previously reported for 14 *S. cerevisiae* strains (15). In particular, premature- (Lys), early- (Asp, Thr, Glu, Leu, His, Met, Ile, Ser, Gln, and Phe), and late- (ammonium, Val, Arg, Ala, Trp, Gly and Tyr) consumed nitrogen sources were able to be differentiated. Interestingly, the proline concentration at the end of the fermentation was greater than that initially present in the synthetic must.

Compared to *S. cerevisiae*, *Starm. bacillaris* showed very different patterns of assimilation of nitrogen sources (Fig. 3). The *Starm. bacillaris* strains that exhibited the same consumption profile, except for arginine and leucine, lacked the ability to efficiently uptake a wide range of nitrogen compounds. In addition, the concentration of some compounds surprisingly increased during fermentation by *Starm. bacillaris* strains. The possibility of releasing amino acids due to autolysis was discounted due to the limited loss of viability of the cells during the middle-end phases of fermentation (lower than 25%, TableS1). According to

these profiles of consumption / production of amino acids, three clusters were identified. The first cluster included the nitrogen sources consumed by the *Starm. bacillaris* strains: ammonium, lysine, arginine, methionine, tryptophane, glutamine, serine, isoleucine, cysteine and phenylanine. Ammonium, lysine, methionine, tryptophane and arginine (MUT5705) were efficiently (between 50 – 100%) uptaken, with complete exhaustion only for ammonium, while the other compounds were consumed to only 30-40 % of the amount provided in the medium. The second cluster consisted of aspartic acid, histidine, proline, serine, threonine and tyrosine amino acids, for which the concentrations remained constant (or with low changes) throughout the fermentation. The last cluster contained alanine, glutamic acid, glycine, leucine (MUT5705) and valine. These amino acids were produced by *Starm. bacillaris* strains during the growth and stationary phases, with substantial increases in their concentration at the end of the fermentation period. The most marked differences were observed for alanine (increase of approximately 170%), glycine (increase of approximately 100%), and valine (increase of approximately 70%). Moreover, the ability to produce substantial levels of leucine was strain-dependent as an 80% increase in the leucine content was observed throughout MUT5705 fermentation. By contrast, this increase was less than 20% for FC54.

Role of the initial nitrogen concentration in nitrogen consumption. The low consumption of amino acids by *Starm. bacillaris* compared with that of ammonium during wine fermentation appeared to be a specific feature of this species. To further investigate this particular phenotype, the FC54 and MUT5705 strains were grown on synthetic medium SM containing 200 mg N/L of nitrogen as (i) the only ammonium source, (ii) a mixture of amino acids and ammonium or (iii) a mixture of amino acids (Table 4). Interestingly, the growth and fermentation performances of both yeasts were significantly increased when the nitrogen resource was exclusively comprised by amino acids (Fig. 4). By contrast, these characteristics were slightly decreased when ammonium was the sole nitrogen compound provided to support growth. Surprisingly, under these fermentation conditions, higher consumption of total nitrogen was observed compared with fermentation in the presence of amino acids (110-134 mg N/L versus 57-69 mg N/L, respectively), even if less biomass was produced. In addition, most amino acids, apart from arginine, tryptophan, lysine, methionine and cysteine, were released into the medium during the growth. Furthermore, the two strains exhibited very similar profiles of amino acid production / consumption when amino acids were provided as the sole nitrogen source or in a mixture with ammonium. It is noteworthy that alanine, leucine, glycine and valine were produced by *Starm. bacillaris* regardless of the nature of the N-resources.

DISCUSSION

Currently, the use of non-*Saccharomyces* yeasts, such as *Torulaspora delbrueckii*, *Lachancea thermotolerans* and *Starm. bacillaris*, in mixed culture fermentations with selected *S. cerevisiae* strains is considered to be an up-to-date strategy that fulfils two main objectives (1,6). First, due to the ability of non-*Saccharomyces* yeasts to produce high levels of glycerol, mannoproteins, organic acids that contribute to the total acidity, and volatile esters with pleasant notes, these yeasts provide a greater aromatic complexity to wines, increasing their quality (6-7). Some non-*Saccharomyces* yeasts are also characterized by limited production of acetic acid and ethanol during wine fermentation. Among these metabolites, ethanol reduction is of great interest as a consequence of global warming and consumer preference for well-structured and full bodied wines produced from fully matured grapes (4). In this context, recent studies proposed the use of mixed culture fermentations with selected *Starm. bacillaris* and *S. cerevisiae* strains to achieve this objective (18). However, attention must be paid to the nutrient concentration of the medium since the initial growth of non-*Saccharomyces* in these fermentations can drastically reduce their availability and limit the subsequent growth of *S. cerevisiae*, thus increasing the risk of sluggish or stuck fermentations (8). Among nutrients, YAN plays a key role in regulating yeast growth, metabolism and, as a result, the chemical and volatile composition of the wines (27). Consequently, further knowledge of the nitrogen requirements of non-*Saccharomyces* species is needed to improve the use of these yeasts in mixed wine fermentation with *S. cerevisiae*.

Specific features of *Starm. bacillaris* in regard to the management of nitrogen. In this study, focusing on the characterization of nitrogen metabolism by *Starm. bacillaris* in comparison with that by *S. cerevisiae*, we first noted substantial differences between the two species in regard to the amount and nature of nitrogen sources assimilated during fermentation. The main characteristic feature of *Starm. bacillaris* strains was their low assimilation of amino acids during wine fermentation compared with ammonium that was entirely consumed. Interestingly, the concentration of several amino acids did not vary throughout fermentation, while some other amino acids were produced, such as alanine, glutamic acid, glycine, leucine (only for MUT5705) and valine.

Furthermore, differences in the earliest nitrogen sources consumed by the two species were observed. In particular, ammonium, tryptophan and arginine were consumed, in large part, by *Starm. bacillaris* strains but were only up-taken during the late stages of growth by *S. cerevisiae*. On the contrary, other amino acids that were more quickly consumed by *S. cerevisiae*, such as serine or threonine, were not assimilated by *Starm. bacillaris* strains.

Otherwise, comparing fermentations in which nitrogen was only provided in an inorganic (ammonium) or an organic (mixture of amino acids) form revealed that, surprisingly, organic N-compounds supported *Starm. bacillaris* growth more efficiently than ammonium. Overall, these observations led us to hypothesize that there are significant differences in the regulation of nitrogen uptake between *Starm. bacillaris* and *S. cerevisiae* species. In *S. cerevisiae*, two regulatory mechanisms as well as the kinetic characteristics of transporters result in the sequential consumption of nitrogen compounds during the growth phase (15). High-affinity permeases under Ssy1p-Ptr3p-Ssy5 (SPS)-mediated control of transport led to early consumption of amino acids, while the uptake of N-compounds that were consumed late involved transporters that were under nitrogen catabolite repression (NCR) or were regulated by SPS low-affinity permeases (28-29). The pattern of consumption of nitrogen sources by *Starm. bacillaris* reveals the strong inability of this species to uptake most amino acids in the presence of ammonium. The molecular basis underlying the prevention of amino acid uptake by ammonium remains to be identified, but different explanations can be considered, such as less efficient SPS-control methods of amino acid permeases or an inhibitory mechanism mediated by ammonium in *Starm. bacillaris*. Another explanation for the preferential use of ammonium by *Starm. bacillaris* is the use of an additional efficient system for ammonium uptake. In line with this assumption, Marini et al. (30) reported that ammonium can enter yeast cells via simple diffusion and using Mep-independent additional ammonium transport system when ammonium concentration drops down. Finally, it is noteworthy that amino acids better sustain *Starm. bacillaris* growth than ammonium, suggesting that the ability of yeasts to catabolize nitrogen sources to efficiently support growth is unconnected to their capacity for early consumption of these N-molecules, as previously observed in *S. cerevisiae* (14,15).

Distinctive characteristics of *Starm. bacillaris* in central carbon metabolism (CCM). The comprehensive comparison of the consumption / production of amino acids, CCM metabolites and volatile molecules between the two species, as summarized in Fig. 5, showed substantial differences in the flux partitioning of the central metabolic network, highlighting the specificities of *Starm. bacillaris* strains. The low production of ethanol and acetic acid by *Starm. bacillaris* strains compared to that of *S. cerevisiae* reveals the low activity of the acetaldehyde pathway in the non-*Saccharomyces* species. This decrease has large-scale effects on the metabolic fluxes, requiring increased production of glycerol to overcome the lower production of ethanol and to maintain the redox balance of cells (31,32). Furthermore, there is a reorientation of fluxes around the pyruvic acid and GA3P nodes that is in line with a reduced carbon channeling towards the acetaldehyde pathway in *Starm. bacillaris,* with increased

production of pyruvate and amino acids and larger amounts of alcohols derived from this intermediate (alanine, leucine, valine and isobutanol) as well as metabolites from glyceraldehyde-3-phosphate (glycine and glycerol).

Surprisingly, though isoamyl alcohol and isobutanol are derived from the same metabolic pathway (33), only the production of isobutanol was increased. By contrast, the formation of isoamyl alcohol was drastically decreased in the *Starm. bacillaris* strains. Different variations in the production of these higher alcohols by *S. cerevisiae* in response to environmental modifications have been previously reported (33-35). These varying responses according to the nature of the higher alcohol, have been shown to be due to changes in acetyl-CoA availability, which is required for the conversion of α-ketobutyrate (KIB), the precursor of isobutanol, into α-ketoisovalerate (KIV), the precursor of isoamyl alcohol (36). Thus, the strongly reduced formation of isoamyl alcohol by *Starm. bacillaris* species is likely due to a decrease in acetyl-CoA availability, which could be, in turn, explained by the low flux through the acetaldehyde pathway. In agreement with a strong limitation of the intracellular pool of acetyl-CoA in non-*Saccharomyces* species, the formation of all of the volatile esters and acids by *Starm. bacillaris*, which are acetyl-CoA-dependent, is considerably low compared to that by *S. cerevisiae*.

During fermentation, the TCA pathway operates as two branches, and the main role of the oxidative route is to provide precursors for anabolism (24,37). Compared to those of *S. cerevisiae*, the production yields of a-ketoglutaric acid and glutamic acid of the *Starm. bacillaris* were increased by 0.0015 (mg/g) and 1.0-1.5 (mg/g), respectively. By contrast, the formation of succinic acid fell by 0.0015 (mg/g). These variations emphasize a redistribution of fluxes from the TCA intermediate α-ketoglutaric acid towards the formation of glutamate at the expense of succinate in *Starm. bacillaris* strains. This redistribution may either reflect specific management of the nitrogen resource by this species or may instead be explained by the low capacity of *Starm. bacillaris* strains to convert α-ketoglutaric acid into succinic acid.

In conclusion, this study highlighted the specific phenotypic features of *Starm. bacillaris* strains during wine fermentation in addition to their extremely fructophilic character (19). In particular, compared with *S. cerevisiae*, this non-*Saccharomyces* yeast exhibits low activity through the acetaldehyde pathway, which triggers an important redistribution of fluxes through the central carbon metabolic network. Furthermore, the two species differ in regard to their pattern of consumption of the wine complex nitrogen resource and their requirements for nitrogen nutrients. From an industrial perspective, these findings provide new relevant prospects in the field of oenology to improve the quality of wines. Thus, in line with the

metabolic reorientations around the pyruvate and glyceraldehyde-3-phosphate nodes of *Starm. bacillaris*, the use of this species in co- or sequential inoculation with *S. cerevisiae* may allow a decrease in the ethanol and acetate contents of wines, with increased production of glycerol, which may also address a key issue of the winemaking industry in the context of global warming (24, 37). A main challenge for the future will be to further decipher the carbon flux distribution in *Starm. bacillaris* cells underlying the phenotypes obtained. Otherwise, the advantages of using *Starm. bacillaris* can be seen to be the limited nitrogen requirements of the non-*Saccharomyces* yeast and its ability to excrete some amino acids, in particular, branched amino acids, during sequential fermentation with *S. cerevisiae*. The latter may use the released amino acids to sustain its growth or to produce volatile molecules of interest derived from branched N-compounds.

ACKNOWLEDGEMENTS

We thank Pascale Brial, Martine Pradal, Marc Perez and Christian Picou for their technical assistance.

TABLE 1 Initial end final concentrations (mg N/L) of ammonium and amino acids in the synthetic musts used in this study

Nitrogen compound	SMA			SMB			SMC		
	Must	FC54	MUT5705	Must	FC54	MUT5705	Must	FC54	MUT5705
Alanine	nd	16.3 ± 0.2	23.1 ± 0.1	12.2 ± 0.1	26.9 ± 0.2	35.4 ± 0.2	13.5 ± 0.1	29.8 ± 0.2	36.3 ± 0.4
Arginine	nd	nd	nd	62.6 ± 0.2	31.1 ± 1.1	9.9 ± 0.1	73.4 ± 0.1	19.4 ± 0.8	15.7 ± 0.1
Aspartique	nd	0.4 ± 0.2	0.5 ± 0.1	2.9 ± 0.1	2.2 ± 0.1	2.4 ± 0.1	2.7 ± 0.2	2.3 ± 0.1	2.7 ± 0.2
Cystine	nd	nd	nd	0.5 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.2	0.4 ± 0.1
Glutamine	nd	3.0 ± 0.2	4.1 ± 0.2	15.3 ± 0.1	4.4 ± 0.2	7.3 ± 0.2	16.9 ± 0.1	5.1 ± 0.1	6.5 ± 0.7
Glutamique	nd	1.6 ± 0.1	1.7 ± 0.1	5.9 ± 0.1	6.1 ± 0.1	7.6 ± 0.5	6.9 ± 0.1	5.8 ± 0.2	7.6 ± 0.1
Glycine	nd	1.6 ± 0.2	1.9 ± 0.1	1.8 ± 0.1	2.7 ± 0.1	3.5 ± 0.1	2.1 ± 0.1	2.8 ± 0.1	3.3 ± 0.1
Histidine	nd	0.4 ± 0.3	0.3 ± 0.1	4.4 ± 0.2	4.1 ± 0.2	4.1 ± 0.1	5.3 ± 0.2	3.5 ± 0.2	4.4 ± 0.3
Isoleucine	nd	0.3 ± 0.1	0.5 ± 0.2	1.8 ± 0.2	1.1 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	0.9 ± 0.1	1.9 ± 0.2
Leucine	nd	0.3 ± 0.1	0.5 ± 0.2	2.8 ± 0.1	3.4 ± 0.1	5.3 ± 0.2	3.2 ± 0.1	4.7 ± 0.1	5.5 ± 0.1
Lysine	nd	nd	nd	1.7 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	1.9 ± 0.1	0.3 ± 0.2	0.1 ± 0.0
Méthionine	nd	nd	nd	1.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	1.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.0
Phénylalanine	nd	0.2 ± 0.2	0.3 ± 0.2	1.7 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.9 ± 0.2	0.9 ± 0.2	1.3 ± 0.2
Proline	nd	1.2 ± 0.1	1.1 ± 0.2	36.8 ± 0	36.3 ± 0.1	36.8 ± 0.2	41.6 ± 0.1	41.9 ± 0.1	42.6 ± 0.1
Sérine	nd	0.3 ± 0.1	0.4 ± 0.1	5.5 ± 0.1	4.6 ± 0.4	4.1 ± 0.1	6.2 ± 0.1	4.2 ± 0.2	4.6 ± 0.2
Thréonine	nd	0.8 ± 0.1	0.9 ± 0.1	4.7 ± 0.1	4.4 ± 0.1	4.3 ± 0.1	5.3 ± 0.1	4.3 ± 0.1	4.9 ± 0.2
Tryptrophane	nd	nd	nd	11.8 ± 0.1	5.5 ± 0.1	4.1 ± 0.2	17.4 ± 0.2	6.6 ± 0.2	5.2 ± 0.2
Tyrosine	nd	0.1 ± 0.0	0.2 ± 0.2	0.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.2	0.9 ± 0.1
Valine	nd	2.2 ± 0.1	3.8 ± 0.1	2.8 ± 0.3	3.8 ± 0.1	4.9 ± 0.1	3.2 ± 0.1	3.6 ± 0.1	5.2 ± 0.1
NH4	200.3 ± 1.3	30.8 ± 1.2	25.9 ± 4.3	22.9 ± 0.1	nd	nd	nd	nd	nd
Total aa	nd	28.4 ± 0.4	39.1 ± 0.1	177.3 ± 0.6	139.5 ± 1.7	134 ± 0.4	206.1 ± 0.2	137.3 ± 1.3	149.3 ± 0.8
Total YAN	200.3 ± 1.3	90.1 ± 2.1	65.8 ± 4.4	200.2 ± 0.7	139.5 ± 1.7	134 ± 0.4	206.1 ± 0.2	137.3 ± 1.3	149.3 ± 0.8

SMA: 200.3 mg N/L ammonium, SMB: 177.3 mg N/L amino acids and 22.9 mg N/L ammonium and SM: 206.1 mg N/L amino acids.

TABLE 2 Metabolites measured in wines produced by fermentation of synthetic must with *S. cerevisiae* and *Starm. bacillaris* strains.

Metabolites	Strains			Sign.
	Uvaferm BC®	FC54	MUT5705	
Main parameters (g/L)				
Sugar consumption	228.5 ± 0.1c	110.9 ± 0.1a	119.1 ± 0.1b	***
Residual sugars	0.7 ± 0.1a	118.4 ± 0.1c	110.1 ± 0.1b	***
Glucose	0.1 ± 0.2a	107.1 ± 0.1b	106.5 ± 0.1b	***
Fructose	0.6 ± 0.1a	11.3 ± 0.2c	3.7 ± 0.4b	***
Biomass (g/L)	3.89 ± 0.3b	0.12 ± 0.2a	0.10 ± 0.1a	***
Ethanol % (v/v)	12.6 ± 0.3c	5.8 ± 0.1a	6.4 ± 0.1b	***
Glycerol	8.1 ± 0.2b	8.2 ± 0.2b	7.7 ± 0.1a	**
Acetic acid	0.64 ± 0.01c	0.11 ± 0.01a	0.21 ± 0.04b	***
Fumaric acid	0.13 ± 0.01a	0.58 ± 0.02c	0.59 ± 0.04b	***
Pyruvic acid	0.11 ± 0.05a	0.87 ± 0.02c	0.45 ± 0.01b	***
Succinic	0.80 ± 0.04c	0.13 ± 0.02a	0.24 ± 0.02b	***
α-ketoglutaric acid	0.13 ± 0.02a	0.37 ± 0.02b	0.37 ± 0.03b	**
pH	3.31 ± 0.01b	3.06 ± 0.01a	3.11 ± 0.01a	***
Titratable acidity	12.17 ± 0.02a	12.84 ± 0.01b	13.11 ± 0.02c	***
Yields				
Ethanol (% v/v)	55.2 ± 0.1b	52.5 ± 0.2a	52.5 ± 0.2a	***
Glycerol (mg/g)	50.1 ± 0.1a	76.5 ± 0.7c	69.7 ± 0.7b	***
Acetic acid (mg/g)	3.9 ± 0.1b	1.8 ± 0.1a	1.5 ± 0.1a	***
Fumaric acid (mg/g)	0.6 ± 0.1a	7.3 ± 0.4c	4.9 ± 0.4b	***
Pyruvic acid (mg/g)	1.7 ± 0.1a	6.8 ± 0.2c	5.4 ± 0.1b	***
Succinic acid (mg/g)	3.4 ± 0.4b	1.9 ± 0.1a	2.1 ± 0.1a	*
α-ketoglutaric (mg/g)	2.1 ± 0.1a	3.4 ± 0.1b	3.5 ± 0.1b	**

The concentration of sugar at the beginning of experiment was 229.2 g/L (114.7 g/L glucose and 114.5 g/L fructose). The values are means ± standard deviation of three independent experiments. Different superscript Latin letters within the same column indicate significant differences (Sig) between pure and mixed culture fermentations (Tukey-b test, $P < 0.05$). Sign·: *** indicate significance at $p<0.001$. Condition I, II: without and with addition of oxygen. TA: titratable acidity; Yields were calculated when both species consumed 100 g of sugars from the fermenting must.

TABLE 3 Concentration of yeast volatile fermentation metabolites for wines produced by fermentation of synthetic must with *S. cerevisiae* and *Starm. bacillaris* strains.

Compounds (μg/L)	Strains			Sig.
	Uvaferm BC®	FC54	MUT 5705	
Alcohols				
Propanol	4133 ± 286a	7323 ± 533b	7476 ± 823b	***
Methionol	884 ± 50b	124 ± 33a	174 ± 17a	***
2-Methyl-1-propanol	73987 ± 3896a	164509 ± 23550b	147844 ± 17478b	**
2-Phenyl-1-ethanol	3177 ± 298b	381 ± 46a	462 ± 131a	***
3-Methyl-1-butanol	308333 ± 14038b	42043 ± 9252a	52091 ± 13517a	***
∑ *Alcohols*	390516 ± 17583b	214382 ± 20197a	208049 ± 31407a	***
Acetate esters				
Propyl-acetate	15.71 ± 1.13b	0.96 ± 0.11a	0.85 ± 0.01a	***
2-Methylpropyl acetate	35.68 ± 1.33b	2.91 ± 0.04a	3.14 ± 0.21a	***
2-Phenylethyl acetate	33.78 ± 1.20b	0.18 ± 0.03a	0.33 ± 0.44a	***
3-Methylbutyl acetate	154.72 ± 16.22b	0.57 ± 0.11a	0.35 ± 0.01a	***
∑ *Acetate esters*	239.89 ± 19.24b	4.62 ± 0.10a	4.67 ± 0.20a	***
Ethyl esters				
Diethyl succinate	2.36 ± 0.51b	1.14 ± 0.02a	1.33 ± 0.11a	**
Ethyl butanoate	23.24 ± 0.52b	1.96 ± 0.70a	1.46 ± 0.18a	***
Ethyl decanoate	48.31 ± 4.21b	1.37 ± 0.31a	1.15 ± 0.12a	***
Ethyl dodecanoate	24.17 ± 7.70b	2.89 ± 0.04a	2.59 ± 0.53a	***
Ethyl hexanoate	51.2 ± 5.42b	2.73 ± 0.61a	3.49 ± 1.2a	***
Ethyl octanoate	88.77 ± 18b	4.93 ± 0.82a	5.41 ± 0.61a	***
Ethyl 2-methylbutanoate	0.13 ± 0.02b	0.02 ± 0.03a	0.06 ± 0.02a	***
∑ *Ethyl esters*	238.18 ± 22.33b	15.04 ± 1.90a	15.48 ± 1.12a	***
Volatile acids				
Decanoic acid	8.58 ± 1.70b	0.95 ± 0.51a	1.42 ± 1.02a	***
Dodecanoic acid	2.68 ± 0.52b	0.72 ± 0.60a	0.44 ± 0.50a	**
Hexanoic acid	1.93 ± 0.64b	0.26 ± 0.12a	0.37 ± 0.12a	***
Isobutyric acid	0.95 ± 0.12	0.98 ± 0.80	1.03 ± 0.12	NS
Octanoic acid	44.71 ± 8.60b	4.88 ± 0.50a	4.96 ± 0.11a	***
Propanoic acid	8.37 ± 2.30b	1.11 ± 0.10a	1.15 ± 0.13a	***
Valeric acid	18.52 ± 1.43b	2.22 ± 0.21a	2.15 ± 0.24a	***
∑ *Volatile acids*	84.79 ± 14.59b	11.10 ± 2.31a	11.53 ± 1.51a	***

Aroma compounds in wines expressed in μg/L, as mean ± standard deviation of three independent experiments Different Latin letters within the same row indicate significant differences among the wines produced from *S. cerevisiae* and *Starm. bacillaris* strains (Tukey-b test; $p < 0.05$). Sig.: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively.

TABLE 4 Metabolites measured in wines produced by fermentation of synthetic musts with *S. cerevisiae* and *Starm. bacillaris* strains.

Metabolites	FC54			Sign[a]	MUT 5705			Sign[b]
	SMA	SMB	SMC		SMA	SMB	SMC	
Main parameters								
Sugar consumption	78.8 ± 2.3a	84.9 ± 5.9b	103.8 ± 0.1c	**	86.9 ± 0.7a	90.5 ± 2.8b	98.8 ± 7.6c	***
Residual sugars	120.4 ± 2.3c	114.2 ± 5.9b	95.3 ± 0.1a	**	112.2 ± 0.7c	108.7 ± 2.8b	100.4 ± 7.6a	***
Glucose	94.6 ± 0.9	95.1 ± 2.2	94.6 ± 1.2	NS	97.1 ± 1.6b	94.2 ± 2.6a	94.2 ± 1.7a	*
Fructose	25.8 ± 1.4c	19.2 ± 5.2b	0.7 ± 1.0a	**	15.1 ± 2.4b	14.5 ± 3.2b	6.1 ± 5.9a	**
Ethanol % (v/v)	5.1 ± 0.1a	4.9 ± 0.3a	5.9 ± 0.2b	***	4.6 ± 0.1a	5.2 ± 0.2b	5.7 ± 0.4c	**
Glycerol	6.6 ± 0.1a	6.9 ± 0.1b	7.3 ± 0.2c	**	6.8 ± 0.1a	6.9 ± 0.1a	7.4 ± 0.2b	*
Acetic acid	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.03	NS	0.03 ± 0.01	0.01 ± 0.01	0.05 ± 0.04	NS
Fumaric acid	0.59 ± 0.06	0.55 ± 0.01	0.58 ± 0.01	NS	0.56 ± 0.03	0.58 ± 0.01	0.59 ± 0.01	NS
Pyruvic acid	0.93 ± 0.02b	0.79 ± 0.03a	1.00 ± 0.06c	**	0.95 ± 0.01b	0.85 ± 0.01a	0.85 ± 0.06a	***
Succinic	0.33 ± 0.08ab	0.34 ± 0.02a	0.48 ± 0.09b	*	0.30 ± 0.02a	0.31 ± 0.02a	0.43 ± 0.02b	**
a-ketoglutaric acid	0.18 ± 0.01a	0.21 ± 0.02a	0.47 ± 0.04b	***	0.14 ± 0.01a	0.21 ± 0.02b	0.31 ± 0.09c	**
Yields								
Ethanol (% v/v)	65.2 ± 1.1b	58.4 ± 0.3a	57.0 ± 1.5a	***	53.1 ± 0.3a	57.6 ± 1.6b	57.7 ± 0.1b	**
Glycerol (mg/g)	83.8 ± 1.2b	84.5 ± 2.2b	70.1 ± 0.2a	***	77.8 ± 0.1b	75.9 ± 0.4a	75.4 ± 0.1a	**
Acetic acid (mg/g)	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.3	NS	0.4 ± 0.1	0.1 ± 0.1	0.5 ± 0.3	NS
Fumaric acid (mg/g)	7.4 ± 1.0b	6.7 ± 0.1b	5.5 ± 0.1a	*	6.4 ± 0.4	6.4 ± 0.3	6.0 ± 0.6	NS
Pyruvic acid (mg/g)	11.8 ± 0.6b	9.6 ± 0.4a	9.6 ± 0.6a	**	10.9 ± 0.2b	9.5 ± 0.5a	8.7 ± 1.3a	**
Succinic acid (mg/g)	4.2 ± 0.1b	3.2 ± 0.2a	4.6 ± 0.9b	*	3.4 ± 0.2a	3.4 ± 0.5a	4.4 ± 0.5b	*
a-ketoglutaric (mg/g)	2.2 ± 0.2a	2.6 ± 0.3a	4.5 ± 0.4b	**	1.6 ± 0.1a	2.2 ± 0.1b	3.1 ± 0.7c	***

The concentration of sugar at the beginning of experiment was 199.16 g/L (99.23 g/L glucose and 99.93 g/L fructose). The values are means ± standard deviation of two independent experiments. SMA: 200.3 mg N/L ammonium, SMB: 177.3 mg N/L amino acids and 22.9 mg N/L ammonium and SM200C: 206.1 mg N/L amino acid. Different Latin letters within the same row indicate significant differences (a) among the strain FC54 and (b) among the strain MUT 5705 (Tukey-b test; $p < 0.05$). [a,b]Sign: *, **, *** and NS indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively.

FIGURE CAPTIONS

FIG 1 Growth dynamics and evolution of metabolites (glucose, fructose, ethanol and glycerol) during pure culture fermentations in SM200 inoculated with *Saccharomyces cerevisiae* and *Starmerella bacillaris* strains. Data are provided as the mean ± standard deviation of two independent experiments. In general, the data for independent experiments were very similar and low deviation standard is therefore shown.

FIG 2 Consumption of yeast assimilable nitrogen (YAN), amino acids and ammonium during pure culture fermentations in SM200 inoculated with *Saccharomyces cerevisiae* and *Starmerella bacillaris* strains. The residual concentrations of each nitrogen compound are expressed as the percentages of the initial concentrations. Data are given as the mean ± standard deviation of two independent experiments.

FIG 3 Consumption of individual amino acids (19) and ammonium during pure culture fermentations inoculated with *Saccharomyces cerevisiae* and *Starmerella bacillaris* strains. The residual concentration of each nitrogen compound is expressed as the percentage of the initial concentrations. Data are given as the mean ± standard deviation of two independent experiments.

FIG 4 A comparative analysis of the fermentation parameters obtained for both *Starm. bacillaris* strains: (A) Parameters related to growth, (B) parameters related to metabolite production, (C) parameters related amino acid concentrations and (D) parameters related to amino acid consumption and production. Must A: 200 mg N/L of NH_4; Must B: 178 and 22 mg N/L of amino acids and NH_4, respectively; and Must C: 200 mg N/L of amino acids. SMA: concentration of the metabolites at the end of the monitored period after fermentation of Must A, SMB: concentration of the metabolites at the end of the monitored period after fermentation of Must B, SMC: concentration of the metabolites at the end of monitored period after fermentation of Must C.

FIG 5 Intracellular carbon flux distribution of *Saccharomyces cerevisiae* and the *Starmerella bacillaris* strains. By-product yields (Y [mg/g sugar consumed]) and consumption/production of amino acids, isobutanol, isoamyl alcohol for *S. cerevisiae* and the *Starm. bacillaris* strains.

Metabolites were measured after 150 and 300 hours of fermentation for *S. cerevisiae* and the *Starm. bacillaris* strains, respectively. Data are the mean ± standard deviation of two independent experiments. The letters in each column indicate significant differences according to ANOVA and the Tukey-b test ($p < 0.0001$).

References

1. Fleet GH. 2008. Wine yeasts for the future. FEMS Yeast Res. 8:979-995. https://doi:10.1111/j.1567-1364.2008.00427.x.
2. Álvarez-Pérez JM, Campo E, San-Juan F, Coque JJR, Ferreira V, Hernández-Orte P. 2012. Sensory and chemical characterisation of the aroma of Prieto Picudo rosé wines: The differential role of autochthonous yeast strains on aroma profiles. Food Chem 133:284-292. https://doi.org/10.1016/j.foodchem.2012.01.024.
3. Pretorius IS. 2016. Conducting Wine Symphonics with the Aid of Yeast Genomics. Beverages 2:36. https://doi:10.3390/beverages2040036.
4. Mira de Orduña RL. 2010. Climate change associated effects on grape and wine quality and production. Food Res Int 43:1844-1855. https://doi.org/10.1016/j.foodres.2010.05.001.
5. Capece A, Romaniello R, Siesto G, Pietrafesa R, Massari C, Poeta C, Romano P. 2010. Selection of indigenous *Saccharomyces cerevisiae* strains for Nero d'Avola wine and evaluation of selected starter implantation in pilot fermentation. Int J Food Microbiol 44:187-92. https://doi.10.1016/j.ijfoodmicro.2010.09.009.
6. Mate JJ, Maicas S. 2016. Application of non-*Saccharomyces* yeasts to wine-making process. Ferment 2:14. https:// doi:10.3390/fermentation2030014.
7. Padilla B, Gil JV, Manzanares P. 2016. Past and future of non-*Saccharomyces yeasts*: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Front Microbiol* 7:411. https:// doi:10.3389/fmicb.2016.00411.
8. Ciani M, Comitini F. 2015. Yeast interactions in multi-starter wine fermentation. Curr Opin Food Sci 1:1-6. https://doi.org/10.1016/j.cofs.2014.07.001.
9. Vilanova M, Ugliano M, Varela C, Siebert T, Pretorius IS, Henschke, PA. 2007. Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. Applied Microbiol Biotechnol 77:145-157. https://doi:10.1007/s00253-007-1145-z.
10. Varela C, Pizarro F, Agosin E. 2004. Biomass content governs fermentation rate in nitrogen-deficient wine musts. Appl Environ Microbiol 6:3392-3400. https://doi.org/10.1128/AEM.70.6.3392-3400.2004.
11. Camarasa C, Sanchez I, Brial P, Bigey F, Dequin S. 2011. Phenotypic landscape of *Saccharomyces cerevisiae* during wine fermentation: evidence for origin-dependent metabolic traits. PLoS One 6:e25147. https://doi.10.1371/ journal.pone.0025147.
12. Martínez-Moreno R, Morales P, Gonzalez R, Mas A, Beltran G. 2012. Biomass production and alcoholic fermentation performance of *Saccharomyces cerevisiae* as a function of nitrogen source. FEMS Yeast Res 12: 477-485. https://doi:10.1111/j.1567-1364.2012.00802.x.
13. Jiranek V, Langridge P, Henschke PA. 1995. Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. Am J Enol Vitic 46:75– 83.
14. Godard P, Urrestarazu A, Vissers S, Kontos K, Bontempi G, Van Helden J, André B. 2007. Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol 27:3065–3086. https://doi.org/10.1128/MCB.01084-06.
15. Crépin L, Nidelet T, Sanchez I, Dequin S, Camarasa C. 2012. Sequential use of nitrogen compounds by *Saccharomyces cerevisiae* during wine fermentation: a model based on kinetic and regulation characteristics

of nitrogen permeases. Appl Environ Microbiol 78:8102–8111. https://doi.org/10.1128/AEM.02294-12.

16. Urso R, Rantsiou K, Dolci P, Rolle L, Comi G, Cocolin L. 2008. Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. FEMS Yeast Res 8:1053-1062. https://doi:10.1111/j.1567-1364.2008.00364.x.
17. Rantsiou K, Englezos V, Torchio F, Risse PA, Cravero F, Gerbi V, Rolle L, Cocolin L. 2017. Modeling of the Fermentation Behavior of *Starmerella bacillaris*. Am J Enol Vitic 68:378-385. https://doi: 10.5344/ajev.2017.16108.
18. Englezos V, Rantsiou K, Cravero F, Torchio F, Ortiz-Julien A, Gerbi V, Rolle L, Cocolin L. 2016. *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. Appl Microbiol Biotechnol 100: 5515-5526. https://doi: 10.1007/s00253-016-7413.
19. Lemos Jr, WJ, Bovo B, Nadai C, Crosato G, Carlot M, Favaron F, Giacomini A, Corich V. 2016. Biocontrol ability and action mechanism of *Starmerella bacillaris* (synonym *Candida zemplinina*) isolated from wine musts against gray mold disease agent Botrytis cinerea on grape and their effects on alcoholic aermentation. Front Microbiol:1249-1249. https://doi:10.3389/fmicb.2016.01249
20. Englezos V, Giacosa S, Rantsiou K, Rolle L, Cocolin, L. 2017. *Starmerella bacillaris* in winemaking: opportunities and risks. Curr Opin Food Sci 17:30-35. https://doi.org/10.1016/j.cofs.2017.08.007.
21. Wang C, Esteve-Zarzoso B, Mas A. 2014. Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence in situ hybridization. Int J Food Microbiol 191:1-9. https://doi:10.1016/j.ijfoodmicro.2014.08.014.
22. Bely M, Sablayrolles JM, Barre P. 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J Ferment Bioeng 70:246-252. https://doi.org/10.1016/0922-338X(90)90057-4.
23. Delobel P, Pradal M, Blondin B, Tesniere C. 2012. A 'fragile cell' sub-population revealed during cytometric assessment of *Saccharomyces cerevisiae* viability in lipid-limited alcoholic fermentation. Lett Appl Microbiol 55: 338-344. https://doi: 10.1111/j.1472-765X.2012.03301.x.
24. Tilloy V, Ortiz-Julien A, Dequin S. 2014.Reduction of ethanol yield and improvement of glycerol formation by adaptive evolution of the wine yeast *Saccharomyces cerevisiae* under hyperosmotic conditions Appl Environ Microbiol 80:2623-32. https://doi: 10.1128/AEM.03710-13.
25. Rollero S, Bloem A, Camarasa C, Sanchez I, Ortiz-Julien A, Sablayrolles JM, Dequin S, Mouret J.R. 2015. Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. Applied Microbiol Biotechnol 99:2291-2304. https://doi: 10.1007/s00253-014-6210-9.
26. Siebert TE, Smyth HE, Capone DL, Neuwohner C, Pardon KH, Skouroumounis GK, Herderich M, Sefton, MA, Pollnitz AP. 2005. Stable isotope dilution analysis of wine fermentation products by HS-SPME-GC MS. Anal Bioanal Chem 381:937-947. https://doi:10.1007/s00216-004-2992-4.
27. Albers E, Larson C, Liden G, Niklasson C, Gustafsson L. 1996. Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. Appl Environ Microbiol 62:3187-3195.
28. Broack JR. 2012. Nutritional control of growth and development in yeast. Genet 192:73-105. https://doi.org/10.1534/genetics.111.135731

29. Wiame JM, Grenson M. Arst, HNJR. 1985. Nitrogen catabolite repression in yeast and filamentous fungi. Adv Microb Physiol 26:1–88. https://doi.org/10.1016/S0065-2911(08)60394-X
30. Marini AM, Soussi-Boudekou S, Vissers S, Andre B. 1997. A family of ammonium transporters in *Saccharomyces cerevisiae*. Mol Cell Biol 17:4282–4293. https://doi: 10.1128/MCB.17.8.4282.
31. Ansell R, Granath K, Hohmann S, Thevelein JM, Adler L. 1997 The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. EMBO J. May 1;16(9):2179-87.
32. Celton M, Goelzer A, Camarasa C, Fromion V, Dequin S. 2012 A constraint-based model analysis of the metabolic consequences of increased NADPH oxidation in Saccharomyces cerevisiae. Metab Eng 14:366-79. https://doi: 10.1016/j.ymben.2012.03.008. Epub
33. Bloem A, Sanchez I, Dequin S, Camarasa C. 2015 Metabolic Impact of Redox Cofactor Perturbations on the Formation of Aroma Compounds in *Saccharomyces cerevisiae*. Appl Environ Microbiol. 82:174-83. https://doi: 10.1128/AEM.02429-15.
34. Clement T, Perez M, Mouret JR, Sanchez I, Sablayrolles JM, Camarasa C. 2013 Metabolic responses of *Saccharomyces cerevisiae* to valine and ammonium pulses during four-stage continuous wine fermentations. Appl Environ Microbiol 79:2749-2758. https://doi: 10.1128/AEM.02853-12.
35. Cadière A, Ortiz-Julien A, Camarasa C, Dequin S. 2011. Evolutionary engineered *Saccharomyces cerevisiae* wine yeast strains with increased in vivo flux through the pentose phosphate pathway. Metab Eng 13:263-71. https://doi: 10.1016/j.ymben.2011.01.008. Epub 2011 Feb 23.
36. Hazelwood LA, Daran JM, van Maris AJA, Pronk JT, Dickinson JR. 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol 74:2259-2266. https://doi.org/10.1128/AEM.02625-07.
37. Caballero A, Segura A. 2017 The quest for lower alcoholic wines. Microb Biotechnol 10:238-241. https://doi: 10.1111/1751-7915.12594.

Figures

Fig. 1

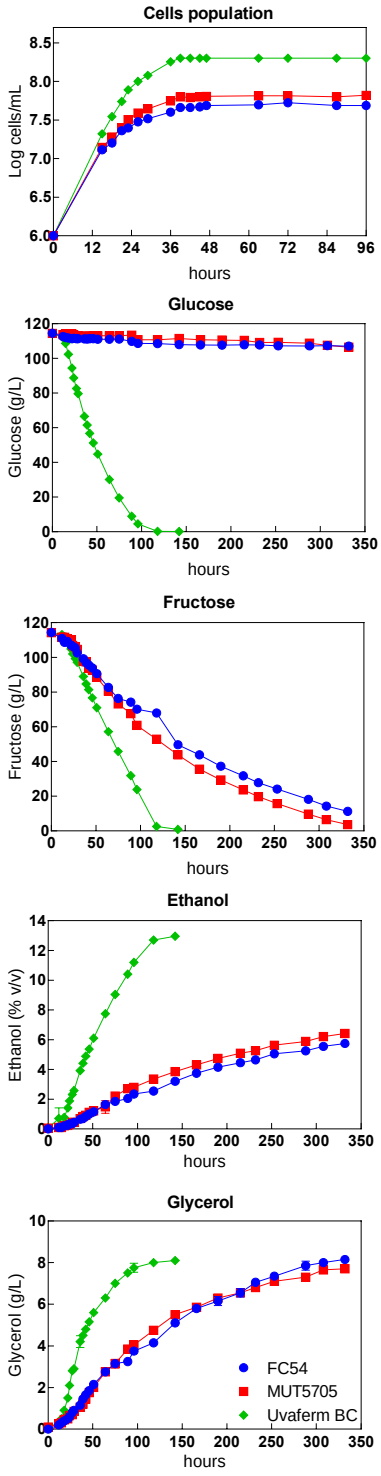


Fig. 2

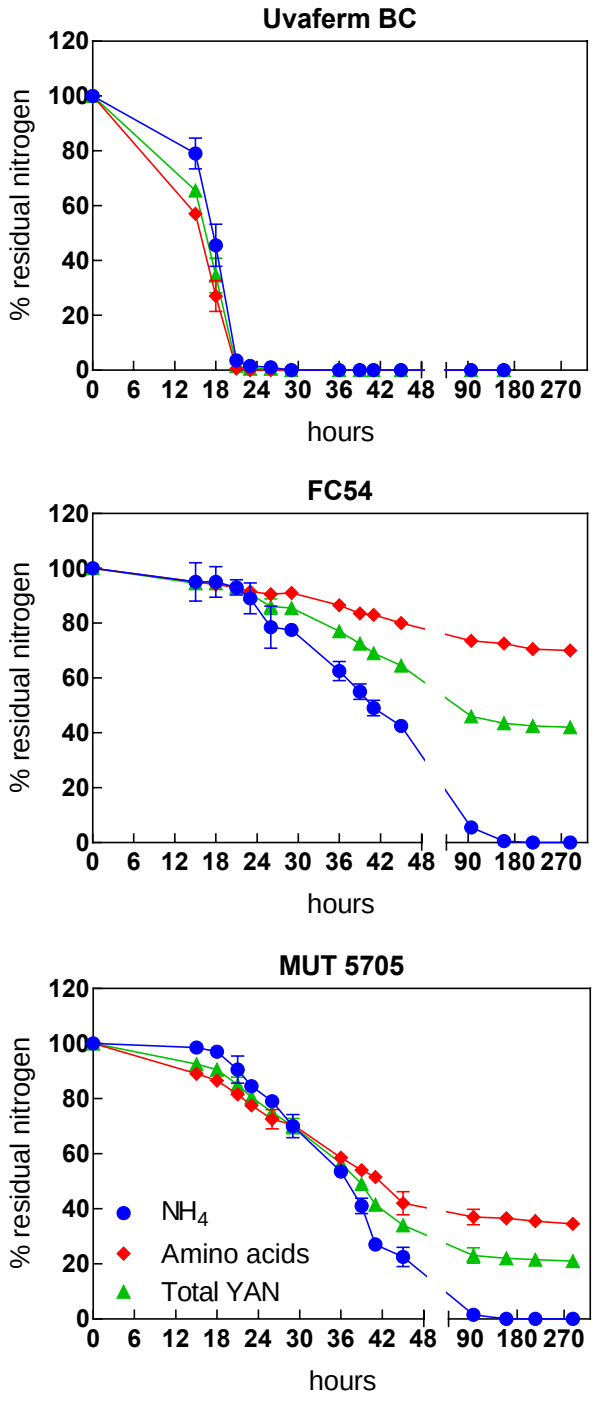

Uvaferm BC
% residual nitrogen
120
100
80
60
40
20
0
0 6 12 18 24 30 36 42 48 90 180 270
hours
FC54
% residual nitrogen
120
100
80
60
40
20
0
0 6 12 18 24 30 36 42 48 90 180 270
hours
MUT 5705
% residual nitrogen
120
100
80
60
40
20
0
NH4
Amino acids
Total YAN
0 6 12 18 24 30 36 42 48 90 180 270
hours

Fig. 3

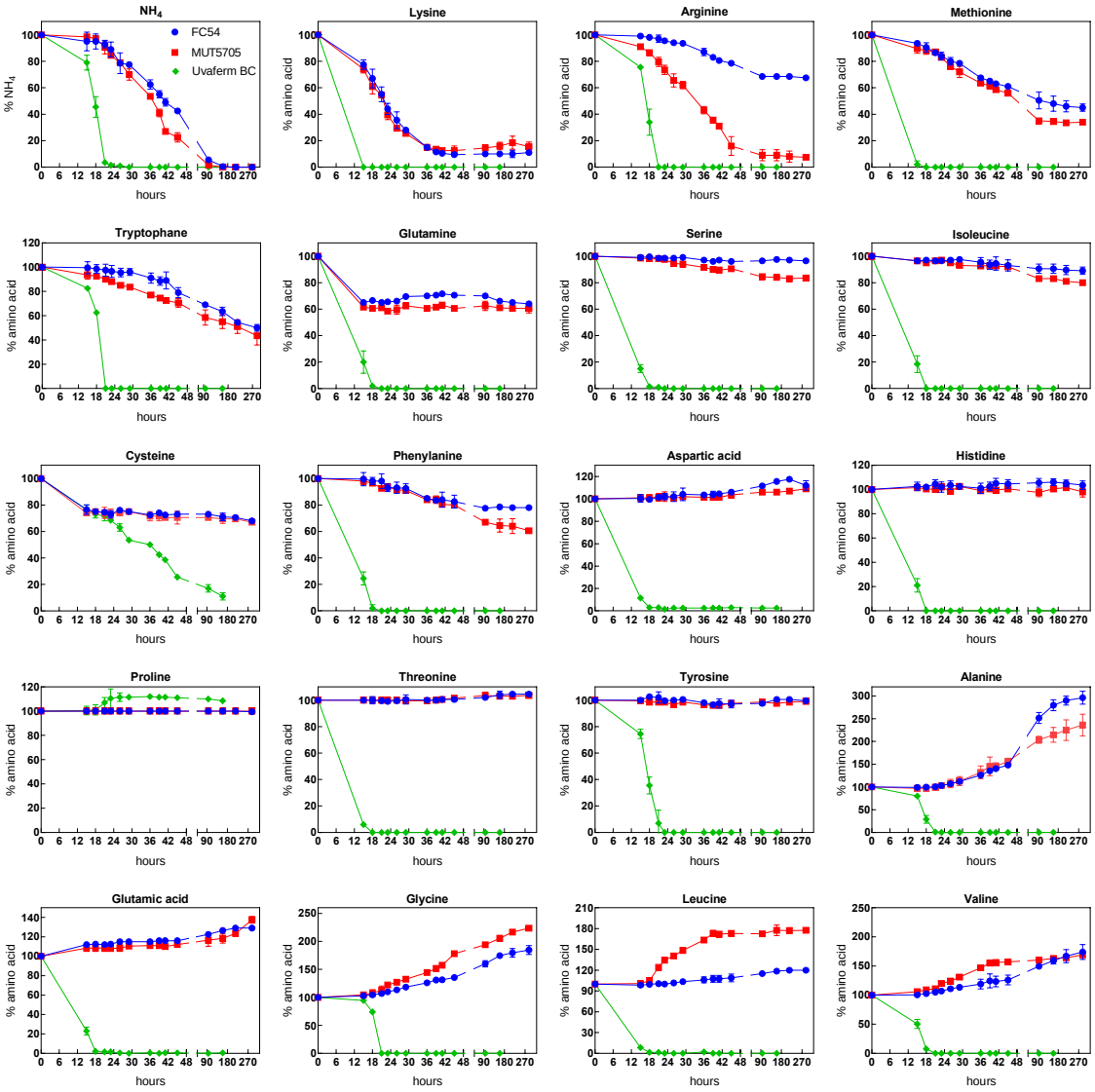
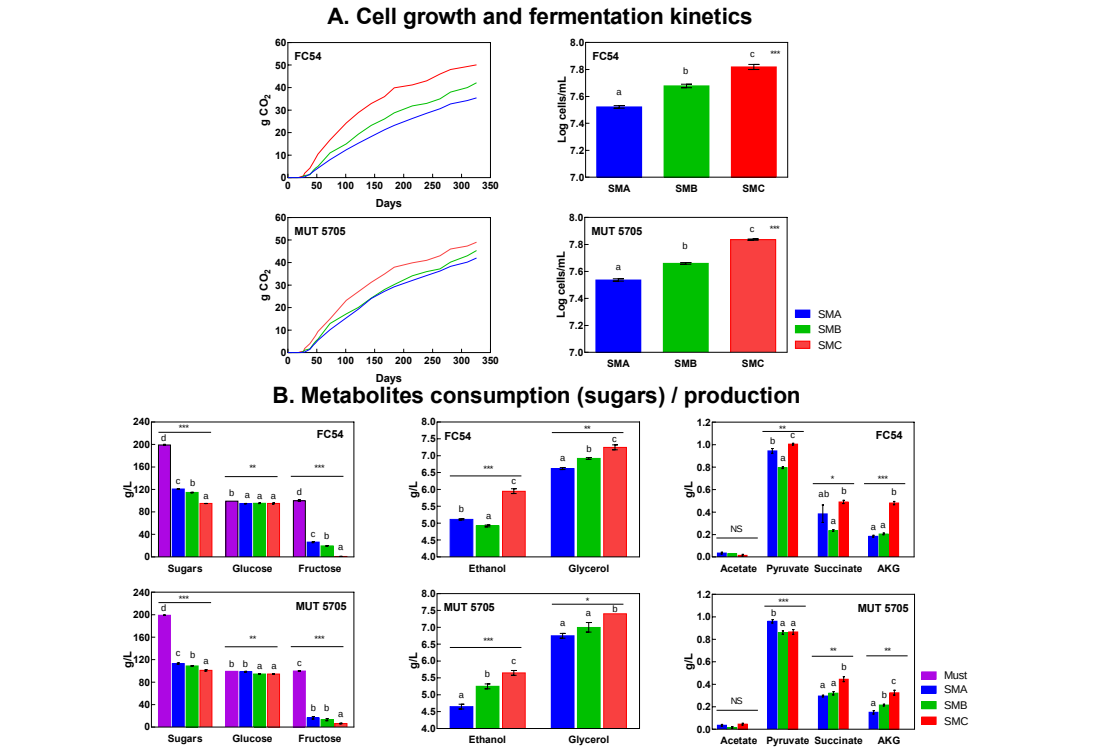

NH4
FC54
MUT5705
Uvaferm BC
% NH4
Lysine
Arginine
Methionine
Tryptophane
Glutamine
Serine
Isoleucine
Cysteine
Phenylanine
Aspartic acid
Histidine
Proline
Threonine
Tyrosine
Alanine
Glutamic acid
Glycine
Leucine
Valine
% amino acid
hours
0 6 12 18 24 30 36 42 48 90 180 270

Fig. 4


A. Cell growth and fermentation kinetics
FC54
MUT 5705
g CO2
Days
Log cells/mL
SMA
SMB
SMC
B. Metabolites consumption (sugars) / production
g/L
Sugars
Glucose
Fructose
Ethanol
Glycerol
Acetate
Pyruvate
Succinate
AKG
Must
C. Amino acids consumption (negative values) / production (positive values)
mg/L
Ala
Arg
Gln
Trp
Asp
Gly
Phe
Ser
Thr
Cys
Lys
Met
Tyr
Glu
His
Ile
Leu
Val