Yeast interaction on Chardonnay wine composition: impact of strain and inoculation time

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Abstract

It is of great importance to understand the molecular characteristics and substantial chemical transformations due to yeast-yeast interaction. Non-targeted metabolomics was used to unravel must in fermentation composition, inoculated with non-*Saccharomyces* (*NS*) yeasts and *Saccharomyces cerevisiae* (*S*) for sequential fermentation. ultrahigh-resolution mass spectrometry was able to distinguish thousands of metabolites and provides deep insights into grape must composition allowing better understanding of the yeast-yeast interactome. The dominance of *S*, characterized by a metabolic richness not found with *NS*, is dependent on inoculation time and on the yeast species present. Co-inoculation leads to the formation of new compounds, reflecting a reshuffling of yeast metabolism linked to interaction mechanisms. Among the modifications observed, metabolomic unravels deep changes in nitrogen metabolism due to yeast-yeast interactions and suggests that the redistribution pattern affects two different routes, the pentose phosphate and the amino acid synthesis pathways.

Keys words: yeast-yeast interaction; sequential fermentation; inoculation time; metabolomics; Chardonnay wine

1 Introduction

Grape must and wine are especially interesting models for considering interactions between microorganisms as they represent a complex microbial ecosystem containing a mixture of different species and strains (Barata et al., 2012). There are many different yeast species typically reported to be associated with the winemaking process but many of them have been studied in the context of spoilage (Morata et al., 2020; Padilla et al., 2016). However, it seems that non-*Saccharomyces* (*NS*) species can have a powerful effect on aroma and flavor formation in wine (Balmaseda et al., 2021; Morata et al., 2020; Sadoudi et al., 2012). The interest for *NS* species is therefore growing, especially for *Torulaspora Delbrueckii* (*D*), *Starmerella Bacillaris* (*B*), and *Metschnikowia pulcherrima* (*P*) (Bordet et al., 2020; Morata et al., 2020; Padilla et al., 2016). An increase in volatile esters has been reported by Renault et al. (2015), using a strain of *D* to obtain more fruity wines (Renault et al., 2015). In addition to its high production of glycerol, *B* is also able to produce aromatic molecules such as lactone, norisoprenoids and terpenols (Sadoudi et al., 2012), as these compounds are characterized as contributing fruity and floral notes to wine. The interest for NS yeasts in winemaking is considerable, however, compared to *S*, the number of studies remains low. Most of the studies still focus on the improvement of aromas using targeted chemical analysis or sensory analysis. *NS* yeasts definitely contribute to the aromatic complexity of the wine (Balmaseda et al., 2021; Jolly et al., 2014; Morata et al., 2020), but the metabolite composition of wines is also of great interest as it can be substantially modified depending on the presence or absence of other microbes (Liu et al., 2016; Roullier-Gall et al., 2020; Sadoudi et al., 2012). Metabolomic appear as an irreplaceable tool for the sensitive analysis of complex samples on a molecular level. In recent years, ultrahigh-resolution mass spectrometry has prevailed as a method of choice in the compositional characterization of utmost complex samples in many scientific disciplines and seems essential for understanding the changes observed linked to interaction mechanisms (Bordet et al., 2020; Petitgonnet et al., 2019; Roullier-Gall et al., 2020).

Individual microorganisms are known to interact with each other, and the types of interaction encountered in mixed populations of microorganisms can be positive, neutral, or even negative. Interactions are usually classified as yeast-yeast cell contact, antimicrobial compounds, competition for substrates, the influence of interactions in gene expression and enzymatic activities, negative-, synergistic- and additive-effect (Ciani & Comitini, 2015). The most obvious example in terms of inhibitory interactions mediated by metabolites with toxic effects is the production of ethanol by *S* above a concentration of 5 to 7% (Heard & Fleet, 1988). The high level of alcohol produced by *S* is the main factor responsible for the dominance of *S* over other *NS* yeasts (Combina et al., 2005). Other factors can lead to selective pressure, in addition to ethanol, including medium-chain fatty acids, acetic acid and even oxygen availability (Fleet, 2003). Recently, antimicrobial peptides corresponding to a fraction of glyceraldehyde 3-phosphate dehydrogenase enzyme have been described as natural biocide secreted by one of the populations present during fermentation (Branco et al.,

2017). It has been reported that some aromatic alcohols such as tryptophol and 2-phenylethanol are used as signal molecules under certain environmental conditions (Avbelj et al., 2016).

Several studies have shown that contact between *NS* and *S* leads to the early death of the *NS* strain, which has not been observed in the context of physical separation of the two populations by different systems such as dialysis membranes (Renault et al., 2013). However, Kemsawasd et al. suggested that cell contact is combined with the secretion of antimicrobial peptides inducing the same phenomenon (Kemsawasd et al., 2015). A recent study of sequential fermentation inoculated with *T* and *S*, conducted with and without cell-cell contact between the two species, showded that cell-cell contact not only affects cell viability but also significantly affects yeast metabolism including volatile and non-volatile profiles (Petitgonnet et al., 2019).

The dynamics of the activity, growth, survival and death of microorganisms during alcoholic fermentation have been the subject of many works (Liu et al., 2017). It has been widely described that the growth kinetics of *NS* in mixed cultures are different from those in simple cultures (Bagheri et al., 2017; Petitgonnet et al., 2019; Roullier-Gall et al., 2020; Sadoudi et al., 2012; Wang et al., 2016). Two different types of yeast inoculation are generally considered in mixed fermentation: co-inoculations and sequential inoculations (Beckner Whitener et al., 2016). Co-inoculation is the simplest way of proceeding in winemaking since all yeasts are added to the must at the same time. In contrast, sequential inoculation requires two distinct steps. *NS* yeasts are added to the must and begin fermentation alone. After a defined period, the *S* yeasts are then added to complete the fermentation. Several authors agree that sequential culture or mixed culture impacts the sensory characteristics of wine (Curiel et al., 2017). According to Wang et al., mixed culture could result in the reduction or disappearance and loss of viability of *NS* yeasts (Wang et al., 2016). The variance between wine from sequential and mixed cultures reflects differences at the yeast metabolic level which have been rarely studied. The whole point of metabolomics is the possibility to study the differences induced by yeast interaction, which in addition can provide information on the nature of the interactions (Roullier-Gall et al., 2020). Although their implementation is more complex, sequential inoculations are particularly interesting because they provide good control over the evolution of fermentation. In this study, the sequential inoculation approach was selected to test the impact of the time of adding *S* on the wine metabolome fermented by three different *NS* yeasts.

2 Material and Methods

2.1 Yeast strains

Three *NS* yeast strains were obtained from the collection of the Burgundy University Vine and Wine Institute. The strains selected for this study were *Starmerella Bacillaris* (*B*), *Torulaspora delbrueckii* (*D*), *Metschnikowia*

pulcherrima (*P*) and a commercial strain of *Saccharomyces cerevisiae* (*S*) used as a reference for sequential inoculation (Supplemental Figure 1). All the yeast strains were grown at 28°C in modified YPD medium (20 gL^{-1} glucose, 10 $g.L^{-1}$ peptone and 5 $g.L^{-1}$ yeast extract with 20 $g.L^{-1}$ of agar for Petri dish cultivation), supplemented with 0.2 $g.L^{-1}$ of chloramphenicol. The yeasts were pre-cultured in 250 mL sterile Erlenmeyer flasks, closed with dense cotton plugs, containing 150 mL of modified YPD medium under agitation (100 rpm) at 28°C for 24h.

2.2 Enumeration of Microorganisms

After yeast growth in YPD medium, 2 microtubes containing one milliliter of yeast culture with 10^6 cells were centrifuged (9000g for 5min). The pellet was suspended in 1mL of in MacIlvaine's buffer (0.1 M citric acid, 0.2 M disodium hydrogen phosphate; pH 4). The first tube was used as control. The second tube was complemented with 2 µL of the viability probe 5-CFDA, AM (5-carboxyfluorescein diacetate, acetoxymethyl ester; Thermo Fisher Scientific) to achieve a final concentration of 1.5 mM incubated for 25 min in darkness at room temperature before flow cytometry (FCM) analysis. The FCM analysis was performed with a BD Accuri C6 flow cytometer. The 5-CFDA (second tube) was excited by the flow cytometer laser at 488 nm and emitted green fluorescence collected by the filter: 530 nm +/- 15nm (FL1 channel). The results were compared to the control tube to eliminate cellular autofluorescence. The data were analyzed using statistical tables that indicate the number and percentage of viable cells as well as fluorescence intensity.

2.3 Fermentations

Fermentations were carried out in duplicate in white must (Chardonnay from Marsannay in France) containing 212 $g.L^{-1}$ of glucose/fructose, a pH of 3.24 and 291 $mg.L^{-1}$ of assimilable nitrogen. The must was centrifuged at 7,000 g for 7 min at 4°C. Sugar concentration and ethanol production were monitored by Fourier transform infrared spectroscopy (FTIR, OenoFOSS™, FOSS, Hilleroed, Denmark). Pure and sequential fermentations were carried out in 250 mL Erlenmeyer flasks, closed with sterile cotton wool, and containing 100 mL of white must. Pure fermentations were inoculated with pre-cultured yeast cells 10^6 $cells.mL^{-1}$ and incubated at 20°C without agitation (B, D, P and S). Sequential fermentations were inoculated with 10^6 $cells.L^{-1}$ *NS* yeast (B, D or P). A second inoculation with 10^6 $cells.mL^{-1}$ *S* was performed 24 h (BS24h, DS24h and PS24h), 48h (BS48h, DS48h and PS48h) and 72h (BS72h, DS72h and PS72h) after the *NS* yeast inoculation.

2.4 Direct infusion FT-ICR-MS

Ultrahigh-resolution FT-ICR-MS was performed with a 12 T Bruker Solarix mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an APOLLO II electrospray source in negative ionization mode.

For the MS analysis, the samples were diluted at 1:100 (v/v) in methanol (LC-MS grade, Fluka, Germany). The diluted samples were infused into the electrospray ion source at a flow rate of 120 µL.h^{-1}. The settings for the ion source were: drying gas temperature 180 °C, drying gas flow 4.0 L.min−1, capillary voltage 3,600 V. The spectra were first calibrated externally by ion clusters of arginine (10 ppm in methanol). The internal calibration of each spectrum was conducted with a reference list including selected wine makers and ubiquitous fatty acids (Roullier-Gall et al., 2014). The spectra were acquired with a time-domain of 4 megawords and 400 scans were accumulated within a mass range of *m/z* 92 to 1000. A resolving power of 400,000 at *m/z* 300 was achieved. Raw spectra were post-processed by Compass DataAnalysis 4.2 (Bruker Daltonics, Bremen, Germany) and peaks with a signal-to-noise ratio (S/N) of at least 6 were exported to mass lists. All exported *m/z* features were aligned into a matrix containing averaged *m/z* values (peak alignment window width: ±1 ppm) and the corresponding peak intensities of all the samples analyzed. Molecular formulas were assigned to the exact *m/z* values by mass difference network analysis using a software tool developed in-house. In total, 3920 detected features could be assigned to distinct and unique molecular formulae. More than 95% of all assignments were found within an error range lower than 0.2 ppm. All further calculations and filtering were performed with Perseus 1.5.1.6 (Max Planck Institute of Biochemistry, Germany) and R Statistical Language (version 3.1.1).

3 Results and discussion

Chardonnay must was divided into 26 aliquots, two were inoculated with pure culture of *S*, eight with *B*, eight with *D* and eight with *P* (supplemental Figure 1). Every 24 hours, *S* was added to two aliquots of each *NS* pure culture (*B*, *D* and *P*) to promote the impact of addition time on sequential fermentation. Different sequential times (24, 48 and 72 hours) were chosen to avoid the rapid dominance of *S*. Samples were collected and analyzed 24, 48, 72 and 96 hours after the beginning of fermentation and at the end of the alcoholic fermentation (corresponding to glucose/fructose concentration lower than 2g.l^{-1}).

3.1 Impact of type of yeast and sequential fermentation on classical oenological parameters

At the end of alcoholic fermentation, all the sequential *S* had an ethanol concentration between 9.0 and 11.5% (*v/v*) and a sugar concentration lower than 2.0 g.L^{-1}. The samples inoculated with *P* took the longest to finish fermentation with at least 12 days in contrast with the pure culture for incomplete alcoholic fermentation. For other *NS* yeasts seven days were enough to complete alcoholic fermentation while it took five days in the case of *S* pure culture (Supplemental Figure 2). Sequential fermentation helps to increase the ethanol concentration but also to reduce the fermentation time compared to *NS* pure culture

fermentation. This is especially obvious when *S* is added early in the fermentation process (supplemental Figure 2 and supplemental Table 1). The decrease in the wine alcohol content from sequential fermentation when compared with *S* pure culture confirmed the utility of *NS* yeast for reducing the ethanol content. For example, *P* pure culture did not exceed an ethanol concentration of 7.8% (v/v) at the end of fermentation (while *S* pure culture reached 11.5%), but reached 9.6%, 10.2% and 10.6% when *S* was added after 72h, 48h and 24h fermentation, respectively (Supplemental Figure 2 and supplemental Table 1). Thus, the earlier *S* is added in the process, the faster the alcoholic fermentation and the higher the ethanol concentration reached. Other classical oenological parameters such as malic acid, volatile acidity and total acidity were also impacted by yeast type and sequential fermentation (supplemental Table 1). As the must and the fermentation conditions were the same for all modalities, differences detected in the final wines compositions and highlight using metabolomics were induced by the type of yeast, the interaction between yeast and the time of S addition.

3.2 Single yeast fermentation markers

Non-targeted direct infusion ultrahigh-resolution mass spectrometry analyses of wines from *S* and each *NS* yeast fermentation were performed at the end of alcoholic fermentation. While the wide range of chemical properties often complicates simultaneous and comprehensive analysis of wine and must, FT-ICR-MS was able to distinguish more than 2000 compounds produced during fermentation. The spectra acquired allowed annotating 2163, 1107 1657 and 759 molecular formulas composed of carbon, hydrogen, oxygen, sulfur, and nitrogen in *S*-, *B*-, *D*- and *P*- wines, respectively (Supplemental figure 3). Differences in composition between wines from *S*, *B*, *D* and *P* could be visualized by comparing raw spectra and are highlighted in the Venn diagram (Figure 1 and supplemental Figure 3). The Venn diagram allows the extraction of 616, 72, 41 and 6 compounds which could be detected exclusively in *S*-, *B*-, *D*- and *P*- wines, respectively (Figure 1). Around 31% of the total composition was unique to one of the wine groups and *S* wines presented the highest number of unique compounds, amounting to 26% of the total composition as unique. These results are rather original, as volatile compounds are usually analysed for yeast strain distinction and, as far as we know, there is no specific aromatic compound linked to one yeast species. Indeed, the differences due to yeast in previous studies were only quantitative (Bordet et al., 2020; Del Fresno et al., 2017). Whereas, wines fermented by *NS* yeasts have mainly been characterized up to now by their volatile profiles, here we confirm the power of the metabolomic approach for in-depth differentiation between species, as demonstrated in our previous results (Roullier-Gall et al., 2020).

Metabolomics is a powerful tool capable of highlighting interspecies differentiation and it also gives details regarding the nature of the differences. Indeed, *NS* species lead to different wine compositions including

unique metabolites (Supplemental Figure 3). Despite these differences in composition, a large part of wine chemical composition is common to at least two wine groups at the end of alcohol fermentation (1651 metabolites representing 69% of the total composition) and 473 metabolites are present in all four groups of wine (20% of the total composition) (Figure 1 and supplemental Figure 3). The annotation of these metabolites covered all the main classes of primary and secondary metabolites including for example carbohydrate, amino acids, organic and fatty acids, nucleotides, vitamins, organosulfur and flavonoid. However, a large proportion of these common compounds are present at different intensities which means at different concentrations. Putative annotated glutarylglycine (level 3), was detected in *S*, *B* and *D* wines at similar intensity whereas malic acid (level 1) was detected in all four groups of wines but present differences in term of concentration (5.2 g/L for S, 4.9 for B, 6.4 for D and 6.3 for P - Supplementary table 1). Finally, only a small part of the composition of the wine samples is present at exactly the same concentration in all the wines (Supplemental Figure 3).

The principal component analysis (PCA) of pure culture wines at the end of alcoholic fermentation confirmed the huge difference in terms of non-volatile composition between *S* wines and *NS* wines (Figure 1). The first component of the PCA explained 70% of the variability between the samples and opposed *S* and *NS* wines. The second component of the PCA explained 16.6% of the variability and highlighted differences between chemical compositions *P*, *B* and *D*. Hierarchical cluster analysis (HCA) confirmed previous results and highlighted the greater proximity between the compositions of *B* and *P* wines. These results reflect considerable differences in yeast metabolism between species, thereby supporting previous analyses (Balmaseda et al., 2021; Rollero et al., 2021). To characterize the impact of yeasts on final wine composition, we computed ANOVA statistics and retrieved a subset of features that showed significant differences in their mean peak intensities (p-value < 0.05) between *S*, *B*, *D* and *P* wines. These extracted features can be used as markers to distinguish and characterize wines depending on the yeast used for the fermentation. 449, 146, 163 and 13 markers were statistically extracted for *S*, *B*, *D* and *P* wines respectively and represented in van Krevelen diagrams (Molecular formulas composed by elements C, H, O, N and S,- Figure 1). The highest number of markers was found for *S* wines (449 markers), confirming the massive impact of *S*, which agrees with previous PCA and HCA. *S* markers are composed of 24% CHO, 14% CHOS, 21% CHON and 41% CHONS compounds located in the van Krevelen diagram, where carbohydrate-, polyphenol- and amino acid-derivatives are expected. For example, out of the 449 markers from S wines, only 32 were putatively annotated (level 3) in known databases. On the 32 annotated compounds, 20 were identify as peptides, one as sulfur containing compounds (vanillic acid sulfate – annotation level 3) and 11 CHO containing compounds. By comparison, *B* markers do not contain any CHOS compounds but are mainly composed of CHON compounds (53%) in the area where amino acid and peptide-like compounds

are expected, while *P* markers contain only nitrogen containing compounds (CHON in orange and CHONS in red) (Figure 1). The differences in terms of composition, especially regarding nitrogen compounds (amino acids and peptides), reflect different species dependent yeast metabolisms. For example it has been reported that the low consumption of amino acids by *B* seems to be a specific feature of this species (Englezos et al., 2018; Gobert et al., 2017). Furthermore, it has been shown that *B* can excrete amino acids during fermentation, including branched amino acids. The fact that P markers mainly contain nitrogen containing markers reflects either a low nitrogen uptake by this species (Roca-Mesa et al., 2020) or/and its known protease activity (C. Snyman et al., 2019). The fact that no CHOS markers were found in *B* might reflect differences in sulfur compound metabolism and point to new studies.

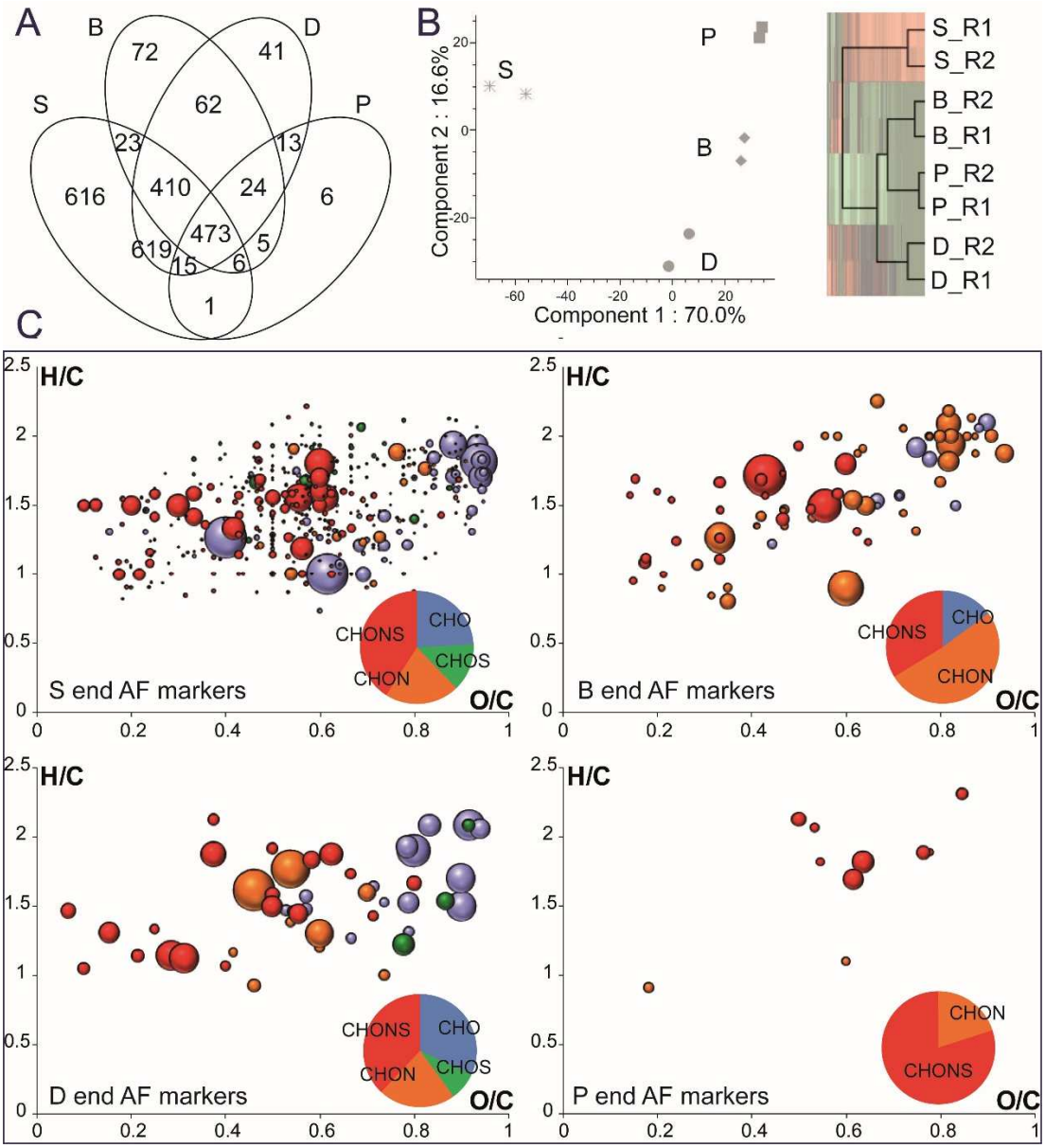

Figure 1: A: Venn diagrams, B: principal component analysis and hierarchical cluster of wines from single yeast fermentations (*S*, *B*, *D* and *P*) at the end of alcoholic fermentation. The first two components represent 86.6% of the variability. The cluster analysis followed by an ANOVA with t-test (p-value 0.05) allows the extraction of specific masses for each single fermentation highlighted in C: the van Krevelen

diagrams. Bubble sizes in the van Krevelen diagrams indicate relative intensities of corresponding peaks in the spectra and bubble colors represent chemical composition with CHO containing compounds represented in blue; CHOS in green; CHON in red and CHONS in orange.

3.3 Metabolic changes during the early stage of alcoholic fermentation

A mass difference network was constructed, based on composition evolution during fermentation (Figure 2 and supplemental Figure 4). *D* sequential fermentations (DS) are represented as examples for visualizing changes in must during the early stages of alcoholic fermentation and compositional modulation due to the time of *S* addition. The nodes represent all the assigned molecular formulas connected to each other by possible chemical transformations during alcoholic fermentation. Using networks, it is possible to make links between the compounds detected (Supplemental Figure 4). It was possible to connect 75% of the annotated compounds using 172 biochemical transformations. For example, hydroxylation (or de-hydroxylation) characterized by a difference between two compounds by an oxygen (+/- O) allows connecting 793 couples of compounds. Hydrolysis or condensation (+/- H_2O) allows connecting 423 couples of compounds, glycine condensation (+/- C_2H_3ON) connected 139 couples of compounds and pyruvic acid decarboxylative addition (+/- C_2H_4O) connected 57 couples of compounds. Networks are colored according to the detection time (24h, 48h, 72h and 92h) of compounds during must fermentation for each pure (*S* and *D*) and sequential fermentation (DS24h, DS48h and DS72h). Networks highlight a clear difference in day-to-day composition between yeast strains (Figure 2). The number of metabolites detected drastically increases with fermentation time. Indeed, the number of compounds started with 388 in must before yeast addition and increased to 1351 compounds for S must and 566 for *D* must in pure culture after 48h fermentation and 2705 for *S* must and 1930 for D must after 96h fermentation (Table 1). A large number of compounds appeared during the first two days of *S* fermentation, with 936 new compounds detected after 48h fermentation (in green) and another 1003 compounds after 72h (in blue), as detailed in Table 1. In contrast, *D* fermentations presented a slower evolution with 546 new compounds detected after 72h fermentation (in blue) and 818 new compounds formed after 96h (in pink). To complete the analysis and statistically quantify the differences in metabolite composition in the fermenting musts, principal component analysis was used. PCA score plots show clear differences in fermentation dynamics between yeast type (*S* versus *NS*), type of fermentation (pure culture vs sequential fermentation) and time of *S* addition in the case of sequential fermentation (24h vs 48h vs 72h) (Supplemental Figure 5, 6 and 7). Based on PCA including all the samples from pure culture (*S*, *D*, *B* and *P*), fermenting musts were rapidly

segregated. For example, after 48 hours fermentation, it was already possible to distinguish the *S* and *D* yeasts based on their metabolic profile (C1 explained 58.2% of the variability –Supplemental Figure 5).

In sequential fermentation the influence of *S* on chemical composition was already visible 48h after its addition (separation from pure culture in PCA). The distinction between pure culture and sequentially fermented must was even more noticeable after 96 hours fermentation, regardless of the *NS* yeast species (Supplemental Figures 5, 6 and 7). The dominance of *S* is extremely rapid and confirmed the excellent adaptation of *S* along the fermentation process. To illustrate this, the PCA showed that the sequential culture of *P* (PS24h) was separated from the pure cultures of *P* by the first component (C1) which represented 91.0% of variability 96h after the beginning of fermentation (Supplemental Figure 6). The same observation was true for the other sequential cultures like BS24h (C1: 77.9% - supplemental Figure 7). Furthermore, when *S* was added 24 hours after the start of *D* fermentation, *S* completely dominated the medium 48 hours after its addition, making it possible to distinguish the sequential culture of *D* alone. The impact of *S* was stronger with early *S* addition. The addition of *S* after 72h fermentation, at DS72h for example, did not impact the composition dynamics as much as for DS24h. Indeed, adding *S* after 24h and 48h fermentation increased the number of new compounds formed (+201 at 48h; +820 at 72h for DS24 and +705 at 72h for DS48) while the addition of *S* after 72h fermentation contributed fewer newly formed metabolites (Table 1). The metabolic richness of *S* was much higher than that of *D* which itself was higher than that of *B* which was richer than *P*. The composition of the must evolved rapidly in the presence of *S*, while the presence of *P* had a lower impact over fermentation time, which reflected a lower metabolic activity specific to *P*.

The number and the type of compounds detected after 96h fermentation showed that a must fermented by a single yeast (*NS* or *S*) has a final composition distinct from the same must fermented by a combination of yeasts (BS, DS and PS). Indeed, the inoculation of *S* for sequential fermentation after 24h fermentation drastically changed the fermentation dynamics and increased the number of newly formed compounds. Thus, sequential DS24h fermentations were closer to the composition dynamics of *S* than to *D* in pure culture, confirming the rapid dominance of *S* over *D*. From the metabolomic viewpoint, these results confirm the ability of *S* to outcompete other microbial species during alcoholic fermentation processes (Albergaria & Arneborg, 2016). The metabolite composition of DS24 was close to that of pure *S*, demonstrating that despite the development of *D* 24h before the addition of *S*, once inoculated, *S* could displace *D*. This ability is linked to its higher fermentation fitness compared to other species (Williams et al., 2015) and to interactions between these microorganisms (Bordet et al., 2020). Our results demonstrate that *S* took over from *D* due to its competitive capacity and physiological fitness. They also clearly show the

existence of negative interactions between these species, confirming previous results (Albergaria & Arneborg, 2016). This highlights that metabolomics is an interesting tool for unraveling yeast interaction and studying yeast dominance.

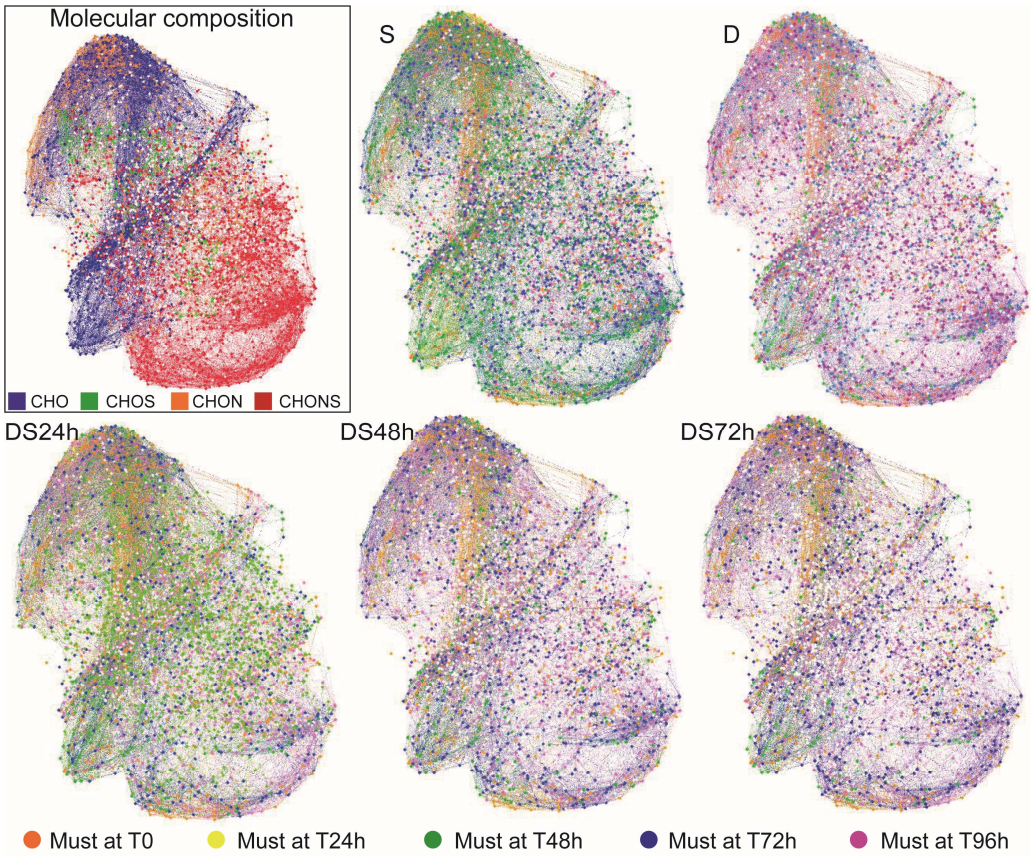

Figure 2: Mass difference network based on the evolution of composition during fermentation in samples *D*, *S* and DS. 75.0% of assigned molecular formulas could be connected in the network by allowing a set of 172 biological transformations. The transformations are non-directed and correspond to edges in the graph. The compounds correspond to nodes in the graph. In the first networks, nodes are colored according to their chemical composition: CHO in blue, CHOS in green, CHON in orange and CHONS in red. In the other five networks, nodes are colored according to their detection time during fermentation. Compounds already present in must are represented in orange, then compounds detected after 24h fermentation are represented in yellow, after 48h in green, after 72h in blue and after 96h in purple.

Table 1: Compounds detected in the initial must and new compounds detected at 24h, 48h, 72h and 96h after the beginning of alcoholic fermentation in samples from fermentations *D*, *S* and DS.

	S	**DS24h**	**DS48h**	**DS72h**	**D**
0h	388	388	388	388	388
24h	27	6	6	6	6
48h	936	201	172	172	172

72h	1003	820	705	546	546
96h	351	611	443	563	818
Total	2705	2026	1834	1675	1930

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332 3.4 Impact of *S* addition time on the final wine

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334 Depending on the yeast and fermentation type (sequential or pure culture fermentation), the evolution of
335 composition during alcoholic fermentation did not lead to the same final wine composition. Hierarchical
336 cluster analyses (HCA) that included all fermentation conditions for each *NS* yeast at the end of the
337 alcoholic fermentation were performed (Figure 3). HCA confirmed that *NS* wines in single or sequential
338 fermentation and *S* wines presented a different composition at the end of fermentation with excellent
339 separation. Sequential fermentation with the addition of *S* at 24h appeared to have the exo-metabolome
340 closest to *S* regardless of the *NS* yeast used for fermentation (*D*, *B* or *P*). These results highlight that the
341 addition time of *S* (24h vs 48h vs 72h) significantly impacts the wine exo-metabolome. The greater
342 proximity between wine from *S* fermentation and sequential fermentation at 24h compared to *NS* single
343 fermentation indicated the rapid dominance of *S* over *NS*, resulting in a high number of unique metabolites
344 and higher intensities of compounds when *S* is added shortly after the beginning of fermentation
345 (Supplemental Figures 8, 9 and 10). Conversely, sequential fermentation when *S* is added at 48h and 72h
346 showed a better balanced impact between *S* and *NS* on the final composition, with a new profile including
347 compounds from *S* and *NS* with compounds unique to sequential fermentation (Supplemental Figures 8, 9
348 and 10). Compounds whose intensity varied significantly (ANOVA with p value 0.05) according to the *S*
349 addition time were then extracted and represented in Van Krevelen diagrams together with a pie chart
350 indicating the composition of the molecular formulas (Supplemental Figures 8, 9 and 10). Each metabolite
351 was classified depending on the composition of their formula into one of the principal chemical families
352 including amino acid and peptides, amino sugars, carbohydrate, lipids, nucleotides, polyphenolic
353 compounds and derivatives and unknown compounds. The highest intensity S metabolites with an
354 intensity decreasing from S to DS72, DS48, DS24h and D were composed of a relative equivalent number of
355 CHO (blue) and CHONS (red) compounds in the mass range of 350-550 Da and mostly annotated as
356 peptides (22.9%), carbohydrate (21.8%), lipids (11.45%) and polyphenolic compounds (9.9%) using
357 metabolomic pathways (Supplemental Figure 8). Conversely, compounds higher in intensity in D wines,
358 with an intensity decreasing from D to DS24, DS48, DS72h and S, were composed of a large proportion of
359 CHONS (red) compounds in the mass range of 250-400 and 450-550 Da and mostly identified as amino
360 sugars (26.2%), peptides (24.8%) and carbohydrate (24.3%).

To go further, all the sequential fermentations were compared to corresponding *S* and *NS* pure culture wine (NS/NS24h/S, NS/NS48h/S and NS/NS72h/S) to focus on the impact of time of *S* inoculation on the final wine composition (Figure 4). First, hierarchical cluster analyses were performed and highlighted compositional differences. As in figure 3, whatever the couple of wines considered, higher profile similarity was always found between the *NS* and sequential fermentation (Supplemental Figure 12). Secondly, Venn diagrams were used to extract compounds at each fermentation and the common metabolites, which were further represented in plots representing the distribution of chemical families (CHO, CHOS, CHON and CHONS) and potential molecular families (Figure 4). The association of both yeast species shows the modification of the exo-metabolome which clearly differs from that of *NS* and *S*, and even differs from the addition of the *NS* and *S* metabolomes. Indeed, part of the composition of *NS* and *S* was not found in wines obtained from sequential fermentation and part of the composition of wine obtained from sequential fermentation was unique, confirming interaction between yeasts in sequential fermentation. Such observations have been reported for volatile compounds. Previous studies reported that mixed fermentation resulted in unique volatile profiles that differed from single strain fermentations (Morgan et al., 2020). However, the specific exo metabolomic pattern reported here confirmed what has been found for volatile profiles and supports the view that in mixed fermentation, the metabolic fingerprint is linked to yeast-yeast interaction and not solely to the addition of the exo metabolome of each single strain.

Focusing on the number of unique compounds found in sequential fermentation, 10 to 20 unique metabolites were highlighted for DS wines depending on the time of *S* addition, 91 to 30 for BS wines and 105 to 24 for PS wines. Surprisingly, the number of unique compounds varied differently depending on the couple of yeasts. Thus, higher numbers of unique compounds were found in B and P sequential fermentation wines compared to D. Moreover, the number of unique compounds decreased with the time of *S* addition in B and P but increased in D. These results confirm that each yeast species has a different metabolism and that it is modulated based on yeast interaction during sequential fermentation. Interestingly, the number of common compounds between *NS* and sequential fermentation increased with the time of *S* addition from 108, 129 to 137 for D/DS24h, D/DS48h and D/DS72h, respectively. On the contrary, the number of common compounds between S and sequential fermentation decreased drastically with the time of *S* addition from 169, 50 to 35 for S/DS24h, S/DS48h and S/DS72h, respectively, indicating a lower impact of *S* on the *DS* composition for the inoculation time of 72h. The same was true for B and BS fermentation whereas the number of common compounds between P and PS was stable for all the *S* inoculation times (24h, 48h and 72h). When *S* was added early in fermentation, its impact was strong and dominant compared to that of NS, whereas the later *S* was inoculated, the weaker its impact on

the final wine composition. Annotated compounds in the biosynthesis of amino acid pathways are represented to illustrate the evolution of composition (Supplementary figure 10). Of the compounds that composed the amino acid pathways, 41 were detected in wines (in black) including five compounds detected with increasing intensity from S to D (in red). These compounds are involved in the pentose phosphate pathway, providing precursors for amino acid synthesis and for the production of polyols, which is a specific trait of D. Seven compounds were detected with increasing intensity from D to S (in blue), and all are involved in amino acid biosynthesis pathways (Supplementary figure 11). These changes reflect competition for nitrogen compounds, leading to a considerable redistribution of fluxes through the central amino metabolic network. Indeed, competition for nitrogen compounds in mixed fermentation has been reported (Rollero et al., 2021). As reported previously (Seguinot et al., 2019), competition for amino acids can lead to the depletion of certain amino acids and consequently to extensive changes in the amino metabolism network to use available nitrogen sources. This redistribution pattern might also reflect specific D nitrogen metabolism. Su et al., reported that *D* consumed nitrogen sources are mainly directed towards the de novo synthesis of proteinogenic amino acids (Su et al., 2020). Thus, our results unravel deep changes in nitrogen metabolism due to yeast-yeast interactions and suggest that the redistribution pattern affects two different routes, the pentose phosphate pathway and the amino acid synthesis pathways.

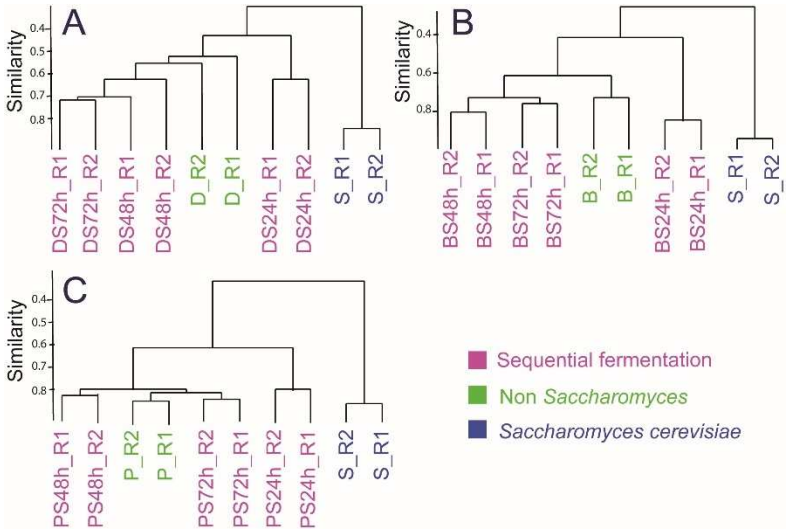

Figure 3: Hierarchical cluster of wine from single yeast fermentations (*NS* in green and *S* in blue) and sequential fermentation (in pink) with *S* added at 24h, 48h and 72h at the end of alcoholic fermentation for *T. delbrueckii* (A), *S. bacillaris* (B) and *M. pulcherrima* (C).

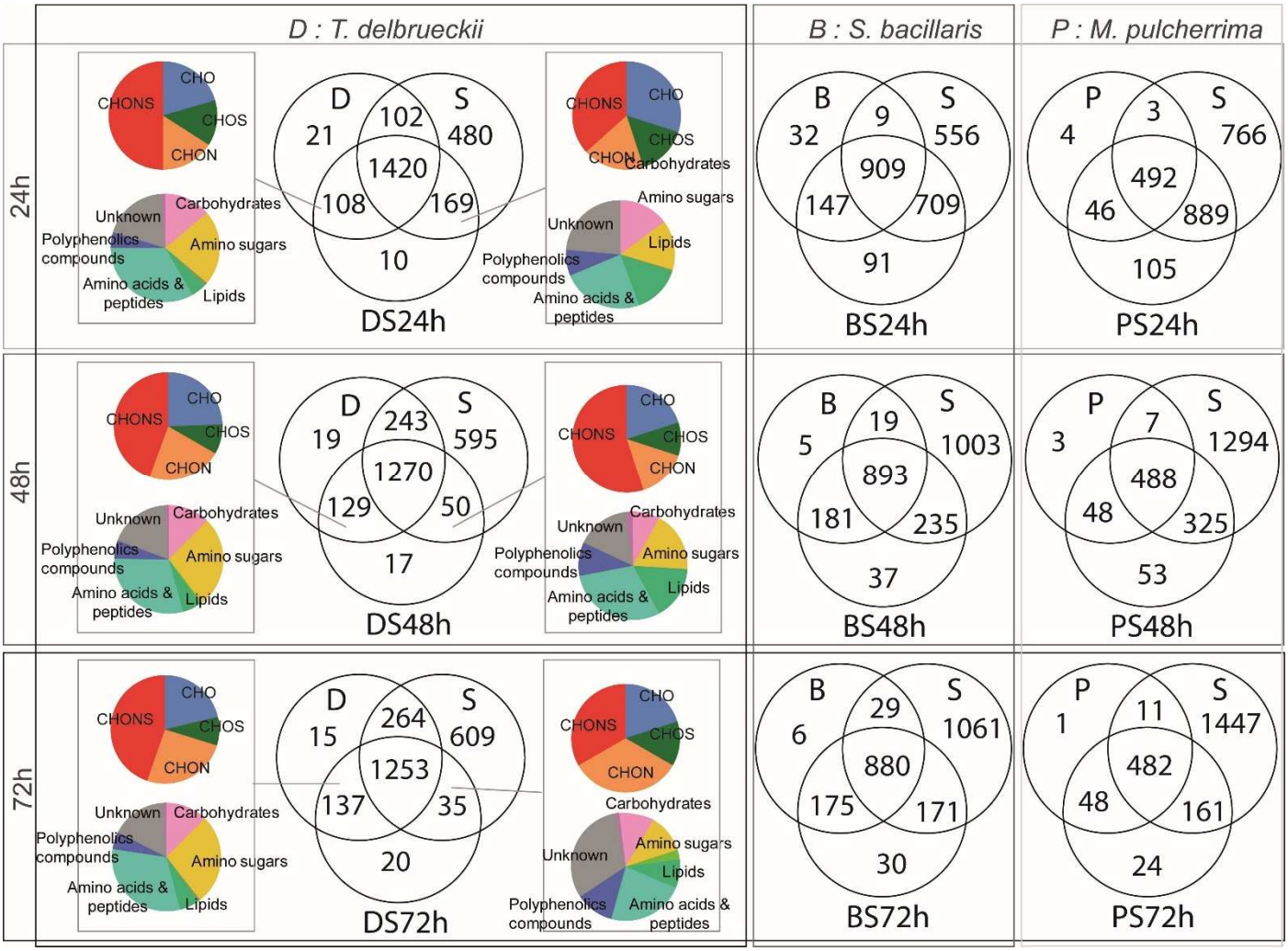

Figure 4: Venn diagrams of wine at the end of fermentation from single yeast fermentations (*NS* and *S*) and sequential fermentation with S added at 24h (NS24h), 48h (NS48h) and 72h (NS72h) with pie charts displaying the compounds common to *NS* and *S* in single and sequential fermentations based on chemical composition (CHO in blue, CHOS in green, CHON in orange and CHONS in red) and metabolic pathway (Carbohydrates in pink, amino sugars in yellow, lipids in green, amino acids and peptides in turquoise, polyphenol compounds in dark blue and unknown in grey). Compounds classified as unknown refer to compounds not annotated in metabolic pathways.

4 Conclusion

Most interaction studies, so far, focused on targeted volatile compounds analysis. However the volatile composition represents only a small part of the modification induced by fermentation. Here we focus on non-targeted ultrahigh resolution mass spectrometry, used to monitored the non-volatile compositional changes, due to yeast interactions and sequential fermentation. Thousands of compounds have been recorded simultaneously and linked to the specific presence of yeast and to their interactions. Based on the exo metabolome, wines from the 4 species could be easily distinguished, reflecting different yeast

metabolisms. Huge metabolic differences have been observed on a larger number than what could ever recorded for volatile compounds. Each yeast led to a different wine non-volatile profile and could be described by a unique pattern of metabolites. From the start of fermentation *S* was characterized by many unique markers, a sign of metabolic richness not found with the other yeast strains (*B*, *D* and *P*). Some strains were characterized by the absence of specific sulfur markers (CHOS) such as *B* and *P*; *P* was characterized by many nitrogen and sulfur containing compounds (CHONS), signs of a specific metabolism. The evolution of the must profile in sequential culture was monitored during alcoholic fermentation, making it possible to demonstrate that the interactions between *S* and *NS* occurred very early, noticeable 24 hours after the addition of *S* in the medium. Once *S* was added in the must, the evolution kinetics of the composition confirmed the metabolic richness of *S* compared to other species with an increasing number of metabolites detected. The ability of *S* to dominate other species is exceptional since it marked the metabolic imprint very quickly as soon as it was present in the medium. Sequential fermentations are very effective in modifying the composition and the final wines are different in terms of the metabolite than the simple addition of pure cultures. Indeed, markers have been highlight as unique to mixed cultures which is the reflection of interactions between yeasts.

Nitrogen metabolism seemed to be the metabolic pathway most altered by sequential cultures, which reflects yeast-yeast interactions. The redistribution pattern particularly affects two different routes, the pentose phosphate pathway and the amino acid synthesis pathways. Indeed, competition for nitrogenous compounds resulted in D by a rearrangement of nitrogen metabolism observable through the evolution of must composition during sequential fermentation. To conclude, this study provides new insights on yeast-yeast interactions during the alcoholic fermentation of Chardonnay must.

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Figure Captions

Figure 1: **Single yeast fermentation discrimination**. Venn diagrams, principal component analysis and hierarchical cluster of wine from single yeast fermentations (S, B, D and P) at the end of alcoholic fermentation. The first two components represent 86.6% of variability. The cluster analysis followed by an ANOVA with t-test (p-value 0.05) allowed the extraction of specific masses for each single fermentation highlighted in the van Krevelen diagrams. The bubble sizes in the van Krevelen diagrams indicate relative intensities of corresponding peaks in the spectra and bubble color represents chemical composition with CHO containing compounds represented in blue, CHOS in green, CHON in red and CHONS in orange.

Figure 2: Evolution of composition during the early stage of fermentation. Mass difference network based on the evolution of composition during fermentation from D, S and DS samples. 75.0% of assigned molecular formulas could be connected in the network by allowing a set of 172 biological transformations. Transformations are non-directed and correspond to edges in the graph. Compounds correspond to nodes in the graph. In the first networks, nodes are colored according to their chemical composition: CHO in blue, CHOS in green, CHON in orange and CHONS in red. In the other five networks, nodes are colored according to their detection time during fermentation. Compounds already present in must are represented in orange, then compounds detected after 24h fermentation are represented in yellow, after 48h in green, after 72h in blue and after 96h in purple.

Figure 3: Impact of the S addition time. Hierarchical cluster of wine from single yeast fermentations (NS in green and S in blue) and sequential fermentation (in pink) with Sc added at 24h, 48h and 72h at the end of alcoholic fermentation.

Figure 4: Impact of sequential fermentation on chemical composition. Venn diagrams of wine at the end of fermentation from single yeast fermentations (NS and S) and sequential fermentation with Sc added at 24h (NS24h), 48h (NS48h) and 72h (NS72h) with a Pie chart displaying the compounds common to NS and S in single and sequential fermentations based on chemical composition (CHO in blue, CHOS in green, CHON in orange and CHONS in red) and metabolic pathway (carbohydrates in pink, amino sugars in yellow, lipids in green, amino acids and peptides in turquoise, polyphenols compounds in dark blue and unknown in grey). Compounds classified as unknown refer to compounds not annotated in metabolic pathways.

Table 1: Evolution of composition during the early stage of alcoholic fermentation. Compounds detected in the initial must and new compounds detected at 24h, 48h, 72h and 96h after the beginning of alcoholic fermentation in samples from D, S and DS fermentations.