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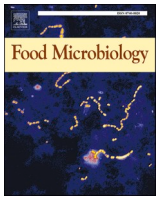

Impact of rare yeasts in *Saccharomyces cerevisiae* wine fermentation performance: Population prevalence and growth phenotype of *Cyberlindnera fabianii, Kazachstania unispora,* and *Naganishia globosa*

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ABSTRACT

Saccharomyces cerevisiae is a highly fermentative species able to complete the wine fermentation. However, the interaction with other non-*Saccharomyces* yeasts can determine the fermentation performance of *S. cerevisiae*. We have characterised three rare non-*Saccharomyces* yeasts (*Cyberlindnera fabianii*, *Kazachstania unispora* and *Naganishia globosa*), studying their impact on *S. cerevisiae* fitness and wine fermentation performance. Using a wide meta-taxonomic dataset of wine samples, analysed through ITS amplicon sequencing, we show that about a 65.07% of wine samples contains *Naganishia* spp., a 27.21% contains *Kazachstania* spp., and only a 4.41% contains *Cyberlindnera* spp; in all cases with average relative abundances lower than 1% of total fungal populations. Although the studied *N. globosa* strain showed a limited growth capacity in wine, both *K. unispora* and *C. fabianii* showed a similar growth phenotype to that of *S. cerevisiae* in different fermentation conditions, highlighting the outstanding growth rate values of *K. unispora*. In mixed fermentations with *S. cerevisiae*, the three yeast species affected co-culture growth parameters and wine chemical profile (volatile compounds, polysaccharides and proteins). *K. unispora* DN201 strain presents an outstanding capacity to compete with *S. cerevisiae* strains during the first stage of wine fermentation, causing stuck fermentations in both synthetic and natural grape musts.

1. Introduction

Wine fermentation is far from being a single-species process. *Saccharomyces cerevisiae* strains –both those naturally present in grape musts and those inoculated in industrial fermentations– coexist with a greatly diverse indigenous community of non-*Saccharomyces* yeasts, especially during the first stages of fermentation (Barata et al., 2012). *S. cerevisiae* is well adapted to the challenging conditions of wine fermentations (high osmolality, low pH, nutrients depletion, presence of ethanol and sulphur dioxide, etc.), and, in most cases, it is able to complete the alcoholic fermentation of grape must sugars, dominating the wine yeast population succession (García-Ríos and Guillamón, 2019; Marsit and Dequin, 2015). Some physicochemical (nitrogen and vitamins availability, pH, temperature, etc) and biological factors (inter-species interactions) of grape musts may compromise the fermentation performance of *S. cerevisiae* strains (Liu et al., 2017; Medina et al., 2012). In particular, some features of certain grape musts, such as a low nitrogen content (Su et al., 2020), vitamins deficiency (Medina et al., 2012), higher pH (Ruiz et al., 2019), and some enological practices such as cold pre-fermentative maceration, display environmental conditions that can reduce the growth fitness of *S. cerevisiae* in favour of other non-*Saccharomyces* yeasts. Thus, some non-*Saccharomyces* species, that are part of the grape must microbiota, can arise during the wine fermentation process, and its metabolic activity can have an important impact on the fermentation performance, by both a direct contribution to the chemical composition of wine (Drumonde-Neves et al., 2021; Roudil et al., 2020), and by modulating the behaviour of *S. cerevisiae* (Ruiz et al., 2020). Different interaction

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mechanisms have been described between non-*Saccharomyces* yeasts and *S. cerevisiae* (Mencher et al., 2021), that can strongly influence *S. cerevisiae* wine fermentation performance and its contribution to wine properties (Ciani and Comitini, 2019).

In the last decade, many studies have explored the impact of several non-*Saccharomyces* yeasts (e.g. *Hanseniaspora, Pichia, Torulaspora, Metschnikowia, Lachancea)* on wine quality (Belda et al., 2017; Jolly et al., 2014; Vicente et al., 2021a; Vicente et al., 2021b, Vicente et al., 2020). However, there is still a lack of knowledge about the role and potential impact of a substantial part of the non-*Saccharomyces* yeasts found in wine environments, which includes a great diversity of species, as revealed by culture-independent studies (Liu et al., 2021). The ease for detection of a large diversity of non-conventional yeasts by meta-taxonomic profiling may allow, in future, to anticipate fermentation kinetic problems or to predict the production of specific aroma-impacting compounds. To do that, it is necessary, not only to detect the presence of these yeasts, but to characterise the impact of such non-conventional yeast species to wine fermentations, studying their interaction patterns with *S. cerevisiae* and their direct and indirect chemical contribution to the resulting wines.

In this work, we have characterised the growth fitness, fermentative potential and enological impact of three wine yeast strains belonging to three non-conventional species: *Cyberlindnera fabianii* (formerly *Pichia fabianii), Kazachstania unispora* (formerly *Saccharomyces unisporus*) and *Naganishia globosa* (formerly *Cryptococcus saitoi*), also attending to some ecological aspects (niche preferences and competition capacity) to understand their interaction patterns with *S. cerevisiae*. In addition, we have studied the population prevalence and average abundance of these genera in a dataset of 272 wine samples coming from different fermentation stages and geographical origins worldwide (de Celis et al., 2022). Although all these three species have been already isolated from grape or wine samples (Drumonde-Neves et al., 2021), very little to no information is available about their role in wine fermentations. We expect our work to bring some light into the biology and enological role of these yeast species and encourage further studies on the metabolic features reported here.

2. Material and methods

2.1. Yeast strains: isolation and molecular identification

In this work, non-*Saccharomyces* wine-related yeasts were randomly isolated from grapes, looking for rare yeast species present in grape musts (Table S1). In brief, grapes samples from eleven different vineyards from several Spanish viticultural regions (Ribera del Duero and La Mancha) were pressed to extract the grape juice to carry out spontaneous fermentations. Liquid samples for yeasts isolation were taken at different stages of the wine fermentation: 0, 24 h and when must density reached 1040 and 999 g/L. Then, an appropriate dilution of musts was spread onto lysine agar plates and incubated at 28 °C for 48 h. The isolates were then identified by sequencing the D1/D2 domain of the 26S large subunit of rRNA gene, using forward NL-1 primer (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and reverse NL-4 (5′-GGTCCG TGTTTCAAGACGG-3′) primers. Sequences obtained by Sanger sequencing were compared by a BLAST search for their taxonomic assignment (NCBI accession numbers to the DNA sequences and taxonomic identity of each isolate are available in Table S1). For this work, we have selected the following three yeast isolates, whose taxonomic identities have been confirmed using the references databases available at MYCOBANK database (in all cases with sequence similarities higher than 99,4% with the type strains): *Cyberlindnera fabianii* LR001 (Cf), *Kazachstania unispora* DN201 (Ku), and *N. globosa* CR112 (Ng), as those belonging to rare and underexplored yeast genera in wine environments. As a control strain for the fermentation trials, we used *S. cerevisiae* Viniferm Diana (AG012) strain (Sc), obtained from the commercial collection of Agrovin S.A (Spain).

2.2. Study of yeast genera prevalence and abundance in wine samples through meta-taxonomics

To determine the population prevalence and average abundance patterns of the genera to which the studied yeasts belong (*Cyberlindnera, Kazachstania, Naganishia,* and *Saccharomyces*), we used the dataset published by de Celis et al. (2022) where a total of 272 wine samples at different fermentation stages (45 grape must; 144 alcoholic fermentation; 51 malolactic fermentation; 32 barrel-ageing) coming from different countries (182 Spain, 47 USA, 31 France, 5 Italy, 5 Denmark, 2 Georgia) were analysed following an ITS-amplicon sequencing strategy (Raw sequences (fastQ) files are available at NCBI (Bioproject: PRJNA814622)).

2.3. Growth phenotypes and environmental preferences

The phenotypic characterization of the yeasts was performed measuring their growth parameters (growth rate and proliferative efficiency) in a panel of 30 culture media (listed in Supplementary Table S2), including 19 fermentation conditions in Synthetic Grape Musts (SGM), and the use of 4 different carbon sources (organic acids) and 7 different nitrogen (organic and inorganic) sources. SGM control medium was prepared as described by Henschke and Jiranek (1993), with some modifications regarding nitrogen content -final yeast assimilable nitrogen was set to 200 mg N/L (60 mg N/L of ammonia-nitrogen ($(NH_4)_2HPO_4$) and 140 mg N/L of amino acids). The growth capacity in different carbon sources were assayed in Synthetic Medium (SM) prepared with Yeast Nitrogen Base 0.17% (BD Difco™, USA), and 0.1% of the corresponding carbon source. Likewise, nitrogen source conditions were assayed in SM prepared with Yeast Carbon Base 0.17% (BD Difco™, USA), and 200 mg N/L of the corresponding nitrogen source. All media were sterilised by 0.45 μm filtration. Yeast strains were pre-cultivated during 24 h in 250 μL of SGM, SM-Glucose or SM-Ammonia media, for SGM, carbon-source and nitrogen-source assays, respectively. Then, yeast strains were inoculated, at a final concentration of $2{\cdot}10^5$ cells/mL, by triplicate, in 96-well plates filled with 250 μL of each medium. Assays were performed at 25 °C (except for those conditions where temperature was tested as a variable) under orbital shaking at 100 rpm. Culture growth was monitored by measuring optical density (600 nm, with a previous orbital agitation of plates at 300 rpm for 5 s) at different time points during 80 h of culture, using the microplate reader Varioskan Flash Multimode Reader (Thermo Scientific, USA). Growth rate and proliferative efficiency (total variation in cell density) were calculated from growth curves by using *GrowthRates* R package v0.8.4 (Hall et al., 2014), adjusting the growth curves to a Baranyi model (Baranyi and Roberts, 1994).

2.4. Co-culture growth assays

Co-culture growth assays were performed by co-inoculating the studied Cf*, Ng* and *Ku* strains with *S. cerevisiae* AG012 (1:1 proportion, to reach a final concentration of $2{\cdot}10^5$ cells/mL) in SGM-based media (Supplementary Table S2). Sc single culture was also inoculated, as a control, at a final concentration of $2{\cdot}10^5$ cells/mL. Co-culture growth assays were performed as described before for the single cultures' growth phenotypes characterization. Likewise, the growth parameters were extracted from growth curves, and co-culture function was calculated as the logarithm of the ratio between the growth parameter of co-culture and the growth parameter of *S. cerevisiae* single culture.

2.5. Killer activity assays

Killer activity and killer sensitivity of the yeast strains were measured using the method described as follow; strains to be tested for killer activity were inoculated in ~1 cm diameter concentrated zones onto YMA-MB plates, previously seeded with a lawn ($2{\cdot}10^5$ cells/mL) of

the strains to be tested for killer sensitivity. Plates were incubated for 7 days at 20 °C. After that, killer activity was detected by the observation of the halo of inhibition. None of the three non-*Saccharomyces* strains showed killer activity against *S. cerevisiae* AG012; and only *C. fabianii* LR001 showed sensitivity against *S. cerevisiae* AG012 strain.

2.6. Microvinifications in synthetic and white (var. Verdejo) grape musts

Microvinifications were carried out, in triplicates, at laboratory scale (in 100 mL borosilicate bottles containing 90 mL of the corresponding must at 25 °C under orbital shaking at 100 rpm), in both synthetic grape must (SGM) and natural white grape must from *Vitis vinifera* L. cv. *Verdejo* grapes (Vitis International Variety Catalogue (VIVC) prime name: Verdejo Blanco; VIVC number: 12,949). The composition of SGM and Verdejo grape musts is detailed in Supplementary Table S3. Pre-cultures of yeasts were carried out in SGM during 24 h (at 25 °C under orbital shaking at 100 rpm). Yeast inocula were adjusted to reach a final concentration of $1{\cdot}10^6$ cells/mL in single inoculation assays; in mixed inoculations, non-*Saccharomyces* strains were inoculated at $1{\cdot}10^6$ cells/mL while *S. cerevisiae* was inoculated at $1{\cdot}10^4$ cells/mL. Seven different assays (4 single and 3 mixed inoculations) were carried out in each must: single inoculations of *S. cerevisiae* AG012 (Sc assay), *C. fabianii LR001* (Cf assay), *K. unispora* DN201 (Ku assay), and *N. globosa* CR112 (Ng assay); and mixed inoculations of *S. cerevisiae* AG012 and *C. fabianii* LR001 (ScxCf assay), *S. cerevisiae* AG012 and *K. unispora* DN201 (ScxKu assay), and *S. cerevisiae* AG012 and *N. globosa* CR112 (ScxNg assay).

Yeast population dynamics were monitored by colony counting in YMA plates for total yeast concentration, and in lysine medium (Oxoid, Hampshire, UK) for non-*Saccharomyces* yeast concentration. Yeast colonies were counted after 48 h of incubation at 28 °C. Fermentation kinetics were monitored by measuring weight loss every 24 h. Liquid samples (2 mL) were taken at different stages of the fermentation for basic analysis of wine chemical parameters. Fermentations were considered to be finished when the weight loss was less than 0.01 g/day. Once fermentations were finished, cultures were centrifuged (7000 rpm/10 min) and supernatants (final resulting wines) were stored at −20 °C until further analysis.

2.7. Basic wine parameters quantification

The concentration of sugars (glucose + fructose), glycerol, L-malic acid, L-lactic acid, acetic acid, ammonia, and primary amino nitrogen (PAN) were determined enzymatically using a spectrophotometer autoanalyzer (Y15, Biosystems, Spain) at 0 h, 96 h and at the end of the fermentation processes. Ethanol concentration was determined using an Infrared Analyser (Bachus 3 MultiSpec, Tecnología Difusión Ibérica, S.L, Spain).

2.8. Wine aroma compounds analysis

The procedure of extraction and separation of wine aroma compounds used in this work was a modification of the method by Ortega et al. (2001). Briefly, to 10 mL of wine 2.5 g of $(NH_4)_2SO_4$, 40 μl of a standard solution mixture (4-methyl-2-pentanol, 800 mg/L and heptanoic acid, 700 mg/L dissolved in ethanol) and 0.4 mL of dichloromethane (PanReac, Spain) were added.

The samples were agitated at 60 rpm for 90 min at room temperature and then centrifuged for 10 min at 5000 rpm. The upper aqueous phase was discarded and the dichloromethane lower phase was transferred to a GC-vial. The extract (3 μL) was analysed in split mode (5:1, 30 mL/min) by an Agilent GC 6850 (Agilent Technologies, Germany) coupled to a flame ionization detector. The volatile compounds were separated on a HP-FFAP column (30 m × 0.25 mm, 0.25 μm, Agilent). The oven temperature was initially held at 35 °C for 5 min, raised by 7 °C/min to 100 °C and finally raised by 3 °C/min to 220 °C for 2 min. The temperature of the injector and detector were 220 and 250 °C respectively. The flow of helium carrier gas was 1.1 mL/min. Volatile compounds were identified and quantified by comparison with standards dissolved in a synthetic wine (12% (v/v) ethanol, 5 g/L of tartaric acid and pH adjusted to 3.5 with 1 M sodium hydroxide) and analysed following the same procedure.

2.9. Polysaccharide extraction and determination by HRSEC-RID

The samples were processed using the methodology described by Ayestarán et al. (2004). Briefly, 10-mL samples in duplicate were concentrated to a final volume of 2 mL using a vacuum evaporator (Univap 148 100ECH; Progen Scientific, United Kingdom). Total soluble polysaccharides were precipitated by adding 10 mL of cold acidified ethanol (hydrochloric acid 0.3 M in absolute ethanol) and kept at 4 °C for 24 h. The samples were then centrifuged (10,000×*g* for 15 min), and the supernatants were discarded. Finally, the precipitates were dissolved in 1 mL of ultrapure water, frozen to −20 °C, and freeze-dried (Telstar LyoQuest HT40, Spain).

The soluble fractions were analysed by high-resolution size exclusion chromatography (HRSEC) to determine molecular distribution and quantify the polysaccharides obtained from the samples (Ayestarán et al., 2004). The lyophilized samples were resuspended in 1 mL of 50 mM ammonium formate and filtered through 0.22-μm acetate cellulose filters (Merck Millipore), and 100 μL were injected into the chromatographic system. The analyses were carried out in an HPLC Agilent 1200 Series system (Agilent Technologies Inc.) with a refractive index detector (RID). Separation was carried out at 20 °C using two Shodex gel permeation HPLC columns (OHpak SB-803 HQ and SB-804 HQ, 300 mm × 8 mm i. d.; Showa Denko). The mobile phase consisted of an aqueous solution of 50 mM ammonium formate applied with a constant flow of 0.6 mL/min for 60 min and a cell RID temperature of 35 °C.

The molecular weight (MW) distribution of the wine fractions was followed by calibration with a Shodex P-82 pullulan calibration kit (P-5, MW = 5.9 kDa; P-10, MW = 11.8 kDa; P-20, MW = 22.8 kDa; P-50, MW = 47.5 kDa; P-100, MW = 112 kDa; P-200, MW = 212 kDa; P-400, MW = 404 kDa; and P-800, MW = 788 kDa) purchased from Waters and four dextrans (BioChemika; 12, 25, 50 and 80 kDa) purchased from Fluka. The polysaccharides were quantified according to the peak area for each fraction using the external standard method with pectin and dextran commercial standards (Sigma-Aldrich) in a range between 0 and 2 g/L ($r^2 > 0.99$).

2.10. Protein determination by HRSEC-DAD

The samples were processed using the methodology described by Canals et al. (1998). Briefly, 15 mL of each sample was dialyzed in duplicate in tubes with an MW cutoff of 3.5 kDa (Membrane Filtration Products Inc.). The dialyzed samples were lyophilized and preserved at −20 °C.

Proteins were analysed by HRSEC to determine molecular distribution and quantify the proteins obtained from the samples (Canals et al., 1998). The lyophilized samples were resuspended in 0.6 μL of 300 mmol/L ammonium acetate and centrifuged (12,000×*g* for 5 min). The supernatant was filtered through 0.22-μm acetate cellulose filters (Merck Millipore), and 100 μL of supernatant was injected into the chromatographic system. The analyses were performed in an HPLC Agilent 1200 Series system (Agilent Technologies) with a diode array detector (DAD) to monitor output at 230 and 320 nm. Separation was carried out at 20 °C using an S 165 Shodex gel permeation HPLC column (OHpak 166 SB- 803 HQ, 300 mm × 8 mm i. d.; Showa Denko). The mobile phase consisted of an aqueous solution of 300 mmol/L ammonium acetate applied at a constant flow of 0.6 mL/min for 70 min. The proteins were quantified according to the peak area for each fraction using the external standard method with bovine serum albumin (Sigma-Aldrich) in a range between 0 and 1 mg/mL ($r^2 > 0.99$).

2.11. Statistical analysis

Statistical analysis was performed with the package stats of R software, version 4.1.2 (R Development Core Team, 2019). The Student t-test was performed to compare average values of the growth parameters extracted from the co-cultures assays. Analysis of variance (ANOVA) and Tukey post-hoc tests were applied to compare the concentration values of wine chemical parameters of the different fermentation assays.

3. Results & discussion

With the aim of establish a collection of different yeast species in wine fermentations, in this work we isolated an extensive wine-related yeast collection of 459 isolates (Table S1) including some rare ones. We identified three isolates belonging to three genera (*Cyberlindnera, Kazachstania* and *Naganishia*) that, as far as the bibliography reports, remain understudied in the wine research context. Thus, in this study we have explored i) the population prevalence and relative abundance of these genera in wine, ii) their growth phenotype and competition ability against *S. cerevisiae* in the wine environment and iii) their impact on oenologically-relevant parameters during wine fermentation, both in single and mixed fermentations with *S. cerevisiae*.

3.1. Population prevalence of Cyberlindnera, Kazachstania, and Naganishia species in wine fermentations

In this work, we studied the population prevalence and relative abundance of the studied genera (*Cyberlindnera, Kazachstania* and *Naganishia,* and *Saccharomyces* as the control yeast) in a wide dataset of 272 wine samples analysed through ITS amplicon sequencing (metataxonomic) previously published (de Celis et al., 2022). Fig. 1 shows that *Naganishia* species are widespread among wine samples (prevalence of 65.07%), but *Kazachstania* spp. (prevalence of 27.21%) and *Cyberlindnera* spp. (prevalence of 4.41%) are much less common. It should be noted that Fig. 1 shows relative abundance data from independent samples collected at different stages of wine fermentation. This fact is important, especially in grape must samples, since the presence of filamentous yeast-like fungi in the grape surface can somewhat misrepresent the relative abundance of yeasts detected by ITS surveys. As expected, the three non-*Saccharomyces* genera studied have lower relative abundance values (on average, lower than 1% of fungal populations) than *Saccharomyces* (prevalence of 94.85%; and average relative abundance of 27.4%). Interestingly, in line with our observation in wine samples, *Naganishia* appears as the most widespread yeast genus within the core microbiome of vineyard soils worldwide, as described in a recent global survey (Gobbi et al., 2022). *Naganishia* is a basidiomycetous yeast so, despite its great prevalence and diversity of phylotypes detected in wine samples (104 ASVs), its fermentative capacity and thus, its contribution to the wine fermentation, is expected to be very limited. Contrary, both *Kazachstania* spp. (order *Saccharomycetales,* family *Saccharomycetaceae*) and *Cyberlindnera* spp. (order *Saccharomycetales,* family *Phaffomycetaceae*) are ascomycetous yeasts that, as can be seen in Fig. 2A, have moderate and low fermentation capacity, respectively. *Kazachstania* species have been reported as typically found at low frequency in grape musts, with some species imparting positive aromas to the wine, and some others (among them, some *K. unispora* strains) associated with rancid-harsh aromas (Jood et al., 2017) or usually found (*K. africana*) in damaged grapes (Drumonde-Neves et al., 2021). Although isolated from grape musts in several studies (Mestre-Furlani et al., 2017; Ortiz et al., 2013) very little information is currently available about the fermentative potential of *Cyberlindnera* species. Here we show that, although found in grape musts and post-fermentative stages (malolactic and ageing) in similar, or even higher, relative abundance values than the other non-*Saccharomyces* genera studied in this work, *Cyberlindnera* yeasts are found in extremely low abundance values during alcoholic fermentations, so its role in this stage of winemaking is expected to be negligible, except in cases of stuck and spoiled fermentations.

3.2. Growth phenotype and competitive ability of C. fabianii LR001, K. unispora DN201, and N. globosa CR112 wine-releated yeasts

To characterise the growth phenotype of the three non-conventional yeasts studied, and to compare it with the *S. cerevisiae* control strain, we quantified the growth rate and proliferative efficiency of the strains in a panel of 30 culture conditions (Table S2). Fig. 2A shows the growth rate and efficiency of each strain in the different fermentation conditions assayed in SGM-based media. First, we observed that although *N. globosa* CR112 was originally isolated from grape must, it showed a null growth capacity in all the experimental fermentation conditions assayed.

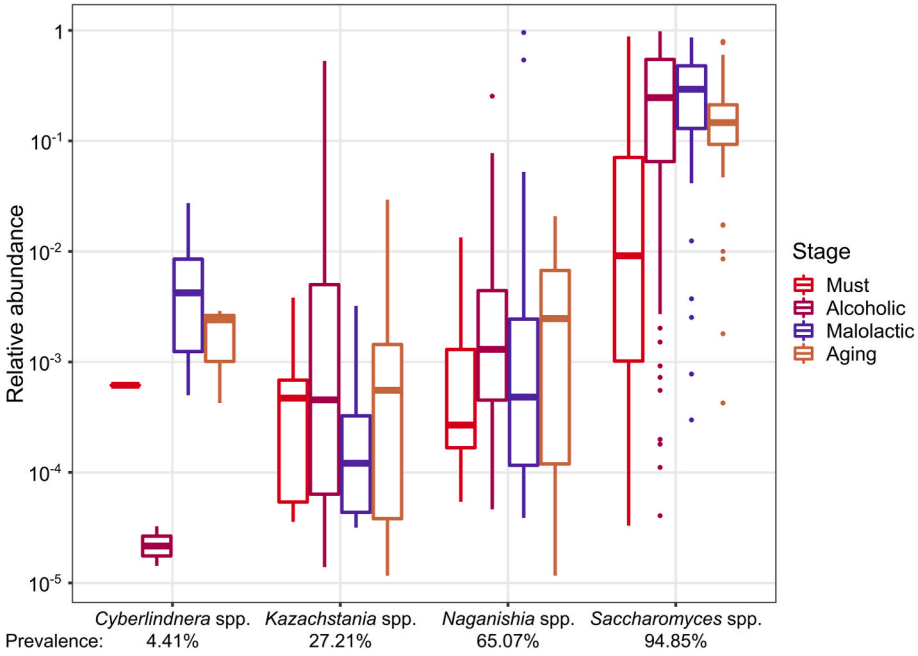

Fig. 1. Distribution of relative abundance values (in boxplots) and population prevalence (percentage of samples in which sequences assigned to these genera are detected) of *Cyberlindnera* (19 ASVs detected), *Kazachstania* (23 ASVs detected), *Naganishia* (104 ASVs detected) and *Saccharomyces* (138 ASVs detected) genera in wine samples collected at different stages (grape musts (n = 45), alcoholic fermentations (n = 144), malolactic fermentation (n = 51), aging (n = 32)), analysed through ITS amplicon sequencing.

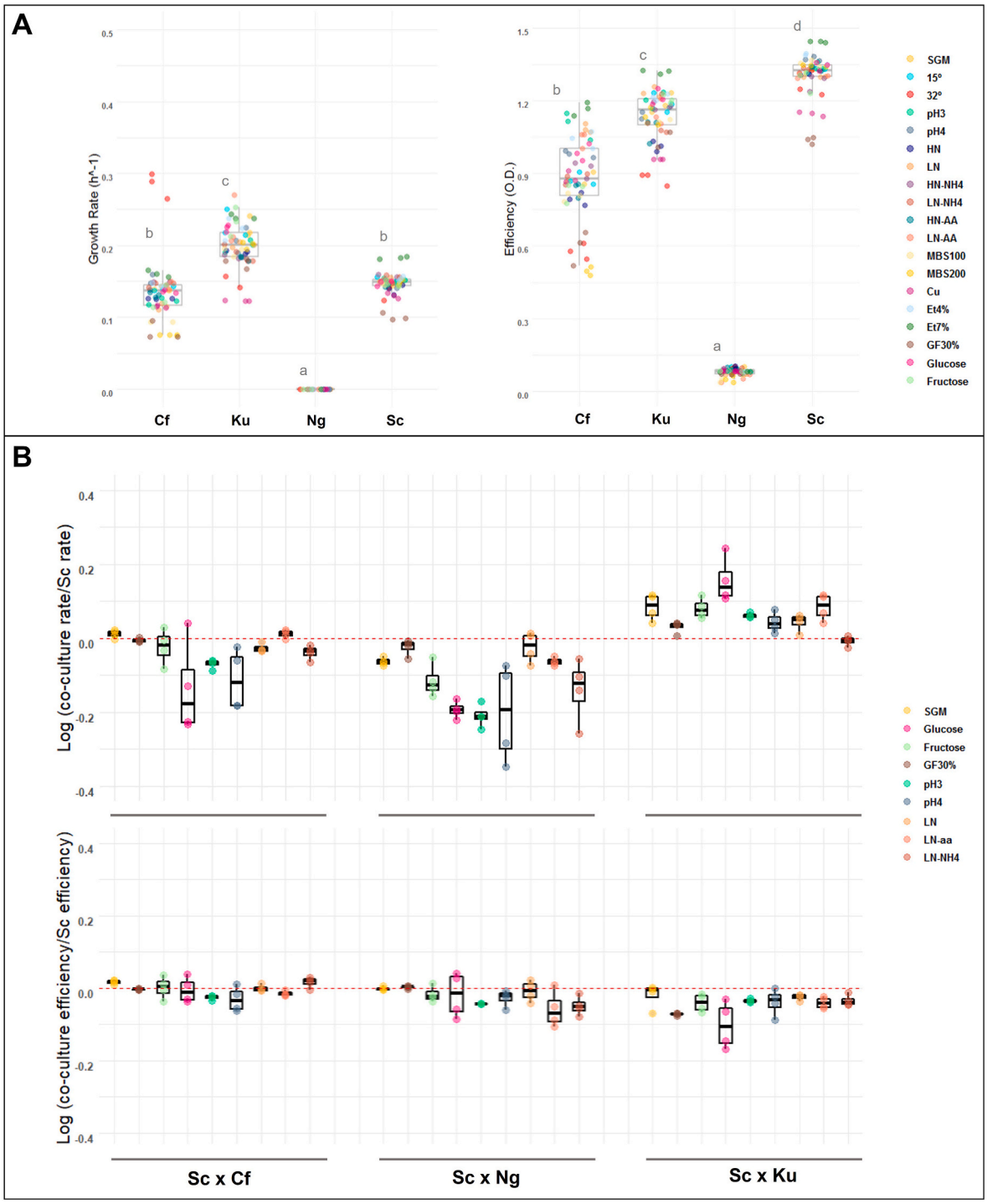

Fig. 2. **A:** Growth parameters of the four yeast strains studied: *S. cerevisiae* AG012 strain (Sc), *N. globosa* CR112 (Ng), *K. unispora* DN201 (Ku) and *C. fabianii* LR001 (Cf). The growth parameters (rate and proliferative efficiency) in all the conditions assayed are represented together in each boxplot, and the individual value for each strain (in triplicates) and condition is represented as a colored dot. Different letters indicate the existence of significant differences ($P < 0.05$) between yeasts in average growth parameters from all wine-based fermentation conditions assayed (the panel of wine-based fermentation conditions assayed is listed in Supplementary Table S1). **B:** Representation of growth parameters of the non-*Saccharomyces* x *S. cerevisiae* co-inoculation assay compared to *S. cerevisiae* single culture in the different wine-based fermentation conditions assayed. Values above the dotted line indicate an increase in the growth parameter in the co-culture assays. Values under the dotted line indicate a decrease in the growth parameter in the co-culture assays. The statistics of global comparison among the three co-culture assays are indicated in Supplementary Table S3.

Bougreau et al. (2019) found that *Naganishia* species (including *N. globosa*, *N. albida*, *N. friedmannii*) were among the yeasts that dominate vineyard environments, mainly in soil but also in berries samples. This could explain its presence in grape musts at the beginning of the fermentation, despite its null growth capacity under wine fermentation conditions. On the other hand, *C. fabianii* LR001 showed a similar growth rate pattern but lower efficiency values than *S. cerevisiae* AG012. Finally, the strain *K. unispora* DN201 showed similar efficiency values but significantly higher growth rates than *S. cerevisiae* AG012.

When yeasts start to grow in grape must –both those inoculated in

industrial fermentations or those native from spontaneous fermentations– they must adapt to the stressful and competitive conditions of this environment. The faster the yeasts adapt to wine conditions and start the exponential growth, the more capacity to compete with other yeasts within the wine environment (Onetto et al., 2021). Thus, we hypothesise that having faster growth rates than *S. cerevisiae* could determine a highly competitive potential of *K. unispora* in wine fermentations. In order to test the ability of the non-*Saccharomyces* yeasts to compete with *S. cerevisiae* in wine fermentations, we co-inoculated *S. cerevisiae* AG012 with the three studied strains under nine different SGM-based experimental conditions (assaying different sugars, nitrogen content and pH values). Fig. 2B shows the growth parameters of the studied co-cultures compared to those of *S. cerevisiae* single-inoculated control assay. The co-inoculation with *C. fabianii* and *N. globosa* (ScxCf and ScxNg assays), caused a negative impact on growth rate, compared with the Sc assay, in almost all fermentation conditions, although we did not observe any significant effect on their final proliferative efficiency (see Supplementary Table S4 for statistics of global comparison). Interestingly, ScxKu co-cultures showed an opposite pattern, with a significant increase in growth rate values and a slight but significant decrease in the proliferative efficiency of the co-culture compared to Sc assay, in almost all fermentation conditions (Table S4). As hypothesised before, the higher growth rates of *K. unispora* DN201 in wine fermentation conditions (Fig. 2A) could compromise the initial development and later dominance of *S. cerevisiae*, which, indeed, showed the higher efficiency values in single inoculations (Fig. 2A). Thus, we argue that a prompt start of the exponential growth followed a higher growth rate, which may determine a faster consumption of nutrients at the initial stages of fermentation (Fig. 3B), may be an important trait to study negative interactions between non-*Saccharomyces* and *Saccharomyces* wine yeasts.

Supplementary Figure S1 represents the growth rate and efficiency of the three non-conventional strains compared to *S. cerevisiae* in assays using different carbon (glucose and organic acids) and nitrogen (ammonia and amino acids) sources commonly found in grape musts. Thus, we can identify potential niches, within the wine environment, that could be preferentially exploited by the non-*Saccharomyces* yeasts studied here. We observed that the *C. fabianii* and *K. unispora* strains

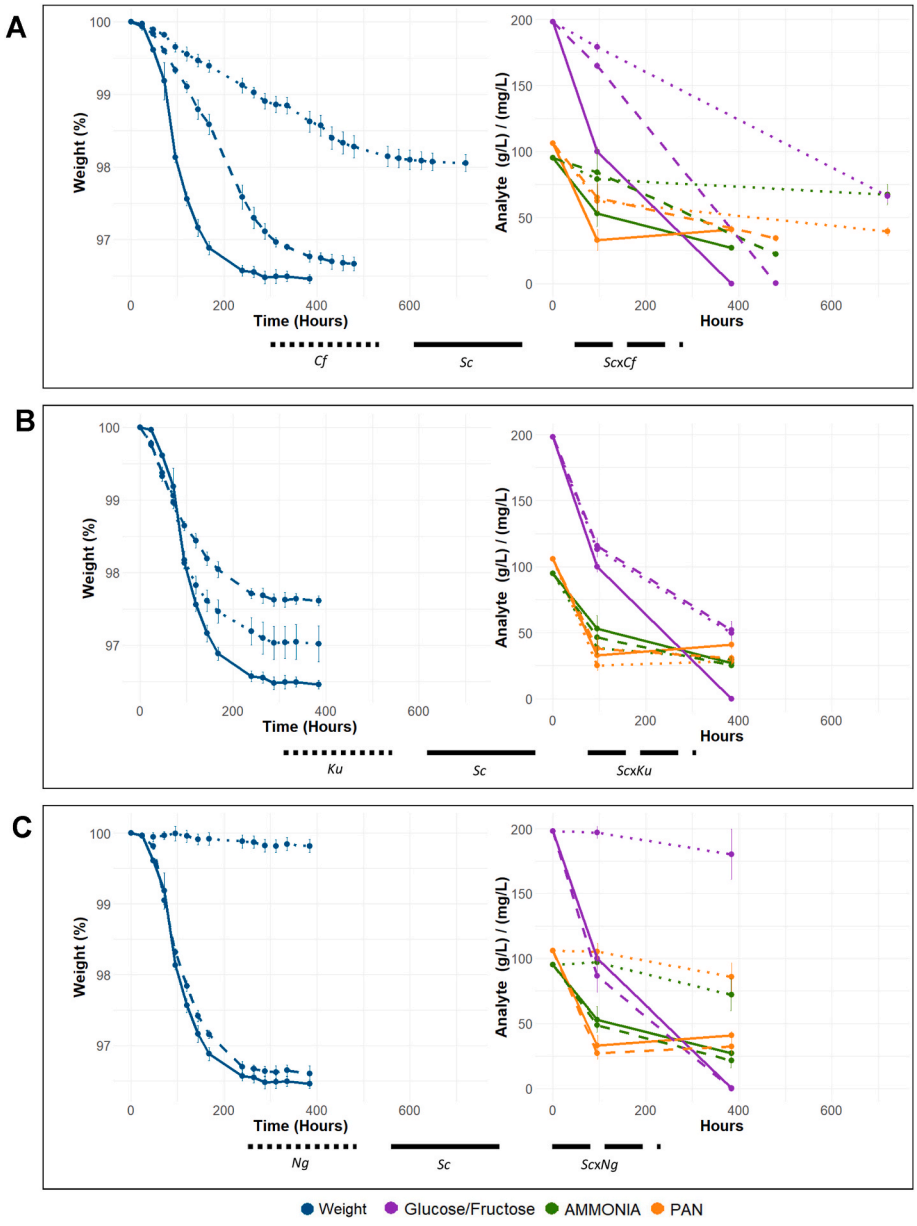

Fig. 3. Fermentation kinetics (weight loss (blue), glucose and fructose (purple), primary amino nitrogen (orange) and ammonia (green)) of single and co-inoculation assays. **A:** Cf (dotted line), Sc (solid line), ScxCf (dashed lines); **B:** Ku (dotted line), Sc (solid line), ScxKu (dashed lines); **C:** Ng (dotted line), Sc (solid line) ScxNg (dashed lines). Data points represent the average value of the 3 replicates with the standard deviation bars. Equivalent results from the assays in natural grape (var. *Verdejo*) must are shown in Supplementary Figure S2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

could grow more efficiently using tartaric and malic acid as sole carbon sources than *S. cerevisiae* AG012. Since malic and tartaric are the predominant acids in grapes, accounting for over 90% of the total acidity in the berry, the capacity of *C. fabianii* and *K. unispora* to consume them efficiently as carbon sources deserve to be explored more in depth for their potential application to modulate wine acidity (Vicente et al., 2020; Vilela, 2019). In addition, malic acid consumption -unlike tartaric acid consumption-is relatively common among non-*Saccharomyces* wine strains (Mateus et al., 2020), and this, among other metabolic features of non-*Saccharomyces* yeasts, it could affect the latter malolactic fermentation performance, as it is the substrate of lactic acid bacteria (Balmaseda et al., 2018). Likewise, we should also highlight that *K. unispora* DN201 showed slightly higher growth parameters values than *S. cerevisiae* when growing with some amino acids (arginine, glutamic acid and lysine) as sole nitrogen sources. Different non-*Saccharomyces* species show specific preferences of amino acid consumption during wine fermentation (Gobert et al., 2017), and the preference and consumption rate of nitrogen sources by non-*Saccharomyces* yeasts can determine the competition for nutrients during wine fermentation, shaping the fermentation performance of *S. cerevisiae* strains. Su et al. (2020) showed that other non-*Saccharomyces* yeasts (*Torulaspora* and *Metschnikowia* strains) also display higher growth capacity than *S. cerevisiae* in poor nitrogen sources (such as histidine or lysine), which can determine a faster depletion of alternative nitrogen sources (Taillander et al., 2014) potentially causing slower or even stuck fermentations.

3.3. Fermentation kinetics and enological impact of C. fabianii LR001, K. unispora DN201 and N. globosa CR112 wine-releated yeasts

Two fermentation matrices, synthetic grape must (SGM) and natural white grape must (var. *Verdejo*), were used to determine the fermentation kinetics, population dynamics and the potential metabolic contribution of the three non-*Saccharomyces* strains studied. We performed both single inoculations (Sc, Cf, Ku, Ng), and co-inoculations of *S. cerevisiae* with the three non-*Saccharomyces* strains (ScxCf, ScxKu and ScxNg) in 1:100 ratio, to mimic the natural condition of grape must microbial communities in which *S. cerevisiae* is underrepresented compared with the rest of the non-*Saccharomyces* yeasts microbiota. It is necessary to clarify that the natural grape must (var. Verdejo) used in this work, although freezed before its use, was not further sterilised, so the indigenous microbiota of this must were not removed. Thus, SGM assays allowed us to describe the metabolic features of the studied yeast strains in experimental conditions, while the fermentation assays performed in natural grape (var. Verdejo) must assays (whose results are shown as part of the supplementary material) allowed us to confirm the most remarkable features of the studied yeast strains in a more complex system, closer to real winemaking conditions.

In SGM, *C. fabianii* LR001 showed a notable fermentation capacity, being able to consume about 67% of the fermentable sugars as a single inoculum (Cf) (Table 1), but it clearly showed slower fermentation kinetics (weight loss rate) than Sc assays (Fig. 3A). In fact, although the mixed fermentation (ScxCf) completed the total consumption of fermentable sugars, its fermentation kinetics were slower at the initial stages of the fermentation -when *C. fabianii* LR001 was the dominant strain-compared to the Sc assay. We should highlight the capacity of *C. fabianii* LR001 to consume malic acid (around 57% of the initial concentration) (Table 1), as we previously demonstrated reporting its notable capacity to grow in malic acid as the sole carbon source (Supplementary Figure S1). This feature is further confirmed looking at the results of the fermentations (both Cf and ScxCf) performed in natural white grape musts, where, although the production of lactic acid suggests the presence and activity of indigenous lactic acid bacteria, only those assays containing *C. fabianii* LR001 showed a complete depletion of the malic acid initially available in the grape must (Supplementary Table S6). Moreover, *C. fabianii* LR001 stands out for its null (undetectable) production of acetic acid in SGM, which results in a decrease in the final concentration of acetic acid in mixed inoculations (ScxCf) compared to Sc in this fermentation condition (Table 1); however, this feature was not reproduced in the fermentation assays in natural grape must (Supplementary Table S6).

Table 1
Main oenological parameters at the end of the SGM fermentation.

	Sc	Cf	*Sc x* Cf	*Ku*	*Sc x Ku*	*Ng*	*Sc x Ng*
Glucose/ Fructose (g/L)	0.03 ± 0.01	66 ± 5 *	0.07 ± 0.06	50 ± 9 *	52 ± 5 *	180 ± 19 *	0.02 ± 0.02
Ammonia (mg/L)	22 ± 9	67 ± 8 *	30 ± 9	22 ± 13	22 ± 6	72 ± 12 *	22 ± 1
PAN (mg/L)	41 ± 3	39 ± 3	31 ± 3 *	28 ± 3 *	32 ± 6	86 ± 11 *	34 ± 2
Glycerol (g/L)	5 ± 0.1	1.0 ± 0.2 *	5.4 ± 0.2 *	7.7 ± 0.1 *	7.8 ± 0.5 *	0.02 ± 0.03 *	4.4 ± 0.2
Acetic acid (g/L)	0.56 ± 0.03	0 ± 0 *	0.33 ± 0.03 *	1.1 ± 0.2 *	1.1 ± 0.2 *	0.44 ± 0.08	0.49 ± 0.03
Malic acid (g/L)	2.3 ± 0.1	1.3 ± 0.2 *	2.3 ± 0.1	2.3 ± 0.1	2.2 ± 0.2	2.7 ± 0.4	2.3 ± 0.0
Ethanol (%; v/v)	12 ± 0	7.6 ± 0.2 *	12 ± 0 [a]	8.9 ± 0.1 *	8.8 ± 0.2 *	1.6 ± 0.0 *	11.9 ± 0.1 *

*Statistical differences (p-value > 0.05) between the assay compared to Sc single fermentation. Lactic acid was measured in all the samples, and its concentration was 0.00 g/L in all cases.

K. unispora DN201 showed a high fermentative capacity and fermentation kinetics -in agreement with its outstanding growth phenotype shown (Fig. 2A)- resulting in a prompt and vigorous start of the fermentation (Fig. 3B). We argue that this feature allows *K. unispora* to initially dominate the population (Supplementary Table 5), and to determine the initial kinetics of the mixed fermentation (ScxKu), as it showed overlapping kinetics with the Ku single inoculation during the initial fermentation stages (Fig. 3B). Later, *S. cerevisiae* seems to drive the fermentation kinetics of the ScxKu fermentation from its intermediate stages (overlapping the weight loss rates of Sc), but then, the fermentation gets stuck, leaving, on average, 51.78 g/L of residual sugars; almost the same concentration than the Ku single inoculation (Table 1), and finding a similar behaviour in the natural grape must fermentation (Supplementary Table S6). Similar results of sugar consumption have been reported by Jood et al. (2017) in single inoculations with other *Kazachstania* species (*K. solicola, K. hellenica* and *K. aerobia*), but in that case none of the studied strains caused stuck fermentations in mixed inoculations with *S. cerevisiae* (Jood et al., 2017; Lin et al., 2020, 2022). Interestingly, *K. unispora* DN201 presents a higher nitrogen:sugar consumption ratio than *S. cerevisiae* AG012 (specially organic nitrogen from amino acids, PAN), as can be detected both at initial stages (96 h) (Fig. 3) and at the end of the fermentation (Table 1). Similar results were found in the fermentation trials in natural grape musts, showing a higher nitrogen consumption of *K. unispora* during the first stages of the fermentation (at 96h; Supplementary Figure S2). However, at the end of the fermentations (Table S6), we find comparable results for the final concentration of inorganic nitrogen (ammonia) but not for the concentration of organic nitrogen (PAN), that showed an opposite pattern than that found in SGM trials. Unfortunately, we did not characterise the amino acid profile of the natural grape musts used in this work, but a different balance in their initial availability, and the release of intracellular amino acids from the yeast cells present in the fermentations arrested in the presence of *K. unispora*, may justify these different results. This competition for nitrogen nutrients has been reported as the reason for sluggish fermentations in co-inoculations with other non-*Saccharomyces* species (Medina et al., 2012). Another interesting fact is

the outstanding production of acetic acid in Ku fermentations, which has been reported as a causative agent in producing stuck fermentations (Mencher et al., 2021; Rasmussen et al., 1995). The production of acetic acid is accompanied by a much higher production of glycerol by *K. unispora* compared to *S. cerevisiae* (see kinetics of production in Supplementary Figure S3), as previously described by Remize et al. (1999). These results, together with the faster consumption of nitrogen (Fig. 3) and the faster population growth of *K. unispora* (Supplementary Table S5) may indicate a different metabolic regulation (increased production of metabolic products from sugar consumption alternative to ethanol production) of this yeast species. The concordance of unfinished fermentations in both synthetic and natural grape musts confirms the ability of *K. unispora* DN201 to causing stuck fermentations, inhibiting not only *S. cerevisiae* AG012 strain but also in the native fermentative microbiota present in the natural must, which is, indeed, able to complete the fermentation in Ng and Cf single inoculations (Supplementary Table S6). Although the specific mechanism by which this species produces stuck fermentations should be further demonstrated, here we argue that the high nitrogen consumption, the high acetic acid production (accompanied with an increased glycerol production), and the faster population kinetics of *K. unispora* DN201, may give it an advantage to compete during the early stages of wine fermentation, causing problems in the latter dominance and activity of *S. cerevisiae*. The production of mycocines or other toxic compounds is another potential explanation but, at least in our conditions, this strain does not present killer activity against the studied yeast strains. Having said that, given that the metabolism of sugars by yeasts depends on dissolved oxygen, we point out that our assays performed at a laboratory scale (100 mL bottles) may differ from the industrial reality on a larger scale, where the availability of oxygen in the fermenting musts is more limited. This fact could change the behaviour of *K. unispora* (as well as other non-*Saccharomyces* yeasts), limiting the consumption and production rates of the mentioned metabolites and its population growth kinetics, and thus, its impact on *S. cerevisiae* performance. Finally, as mentioned before, the inoculation conditions imposed in our trials, where the non-*Saccharomyces* yeasts were inoculated at a population density of $1{\cdot}10^6$ may differ from those expectable to be found in natural wine yeast communities, where a lower population density may limit the effect of the studied species in the fermentation kinetics.

In single inoculation, *N. globosa* CR112 (Ng) showed a very limited fermentation capacity (Table 1), nevertheless, we detected an increase in the population density of this species in both single and mixed fermentations (Supplementary Table S5). In mixed inoculations (ScxNg), *N. globosa* do not significantly impact neither the fermentation kinetics (Fig. 3C) nor the basic composition of the resulting wines, showing similar figures to those of *S. cerevisiae* single inoculations (Sc) (Table 1). Thus, we can conclude that there is not a clear direct or indirect (via interaction with *S. cerevisiae* strain) impact of *N. globosa* CR112 in wine fermentation performance.

3.4. Analytical profile of polysaccharides, proteins, and volatile compounds in the final wines

Polysaccharides, mannoproteins and proteins are the main colloid substances present in white wines (Waterhouse et al., 2016). A portion of these polysaccharides and proteins comes directly from the grapes whereas another portion, especially mannoproteins, is released by yeasts during the alcoholic fermentation and its subsequent autolysis (Pons-Mercadé et al., 2021). These macromolecules have a great interest because they contribute to some interesting wine sensory attributes such as mouthfeel and sweetness (Gawel et al., 2018; Marchal et al., 2011). Moreover, their presence softens the astringency and bitterness of wines (González-Royo et al., 2013), improves both effervescence quality and foam stability (Medina-Trujillo et al., 2017) and, in the particular case of mannoproteins, they can contribute to wine protein (Dupin et al., 2000) and tartaric acid (Moine-Ledoux and Dubourdieu, 2002) stabilization. However, some proteins of grape origin can originate protein haze (Esteruelas et al., 2009).

Fig. 4 shows the concentration of polysaccharides and proteins of the final wines obtained in SGM assays (detailed in Supplementary Tables S7 and S9, respectively). In SGM, Sc released higher concentration of polysaccharides followed in decreasing order by Cf, Ku and Ng. when these yeasts were individually inoculated (Fig. 4A). It must be highlighted that *N. globosa* only released the low molecular weight fraction of polysaccharides (F4, 7.5–1.0 kDa) whereas the other yeasts produced all the fractions (F1, 1100-180 kDa; F2, 180-40 kDa; F3, 40–7.5 kDa and F4, 7.5–1.0 kDa). However, the co-culture assays ScxCf showed an increase of total polysaccharide concentration, especially of the low molecular weight fraction (F4), in comparison with the single Sc inoculation assay. In contrast, a lower release of polysaccharides was detected when *S. cerevisiae* was co-inoculated with *K. unispora* or *Naganishia* sp, in comparison with the single Sc inoculation assay, and it occurs in both SGM (Supplementary Tables S7) and natural grape must assays (Supplementary Table S8).

Ku showed the highest capacity to release proteins in SGM assays (two times higher than *S. cerevisiae*; Supplementary Table S9), followed in decreasing order by Cf, Sc and Ng (Fig. 4B). In general, this behaviour was observed in all the molecular weight fractions (F1, >75 kDa; F2, 75-50 kDa and F3, 7.5–1.0 kDa). The low release of proteins of *N. globosa* is also probably related with its very limited fermentation ability. When co-inoculation assays were performed no significant differences between the individual inoculation of *S. cerevisiae* and co-inoculation with *C. fabianii* or *N. globosa* were observed. In contrast, the presence of *K. unispora* trends to a protein content increase in all the performed assays (both single and mixed fermentations). Despite these results obtained in SGM, the natural grape (cv. Verdejo) must fermentations, which had a much higher protein content than the SGM, showed no statistically significant differences between the inoculation strategies, so the role of these isolates in the proteins profile of wines cannot be totally confirmed in real winemaking conditions (Supplementary Tables S9 and S10, respectively).

Finally, given the importance of the matrix in the release of aroma compounds, just a few common patterns were observed in SGM and natural grape must fermentations (detailed data in Supplementary Tables S11 and S13, and S12 and S14, respectively). In addition, in our study, we should take precautions when comparing data of the volatile profile of the resulting wines, especially in the case of ScxKu, since it has left around 50 g/L of residual sugars at the end of the fermentation, and this have important consequences on the yeast metabolism. The most conserved pattern across fermentation conditions (SGM and natural grape must) in all the non-*Saccharomyces* yeast studied is a significant decrease in the total concentration of fatty acids (SCFA and MCFA), which can be found in both their single and mixed fermentation trials (Fig. 4C, Supplementary Tables S11 and S13). Specifically, in SGM, the three non-*Saccharomyces* yeasts produce a much lower concentration, and reduce the concentration in the mixed fermentation assays, of 2-methylpropanoic acid and butyric acid (as SCFAs), and hexanoic acid and octanoic acids (as MCFAs) compared to *S. cerevisiae* single assay. These compounds can be related to cheesy or rancid butter aromas when they are found in wine at moderate high concentration. Other non-*Saccharomyces* species, such as *Metschnikowia pulcherrima,* have shown similar results of reducing the concentration of fatty acids (Ruiz et al., 2018).

Regarding the production of higher alcohols, *K. unispora* stands out for its high production of 2-phenylethanol and 2-phenylethyl acetate, reaching a total concentration of higher alcohols and higher alcohol acetates in Ku assays two times higher than that produced by *S. cerevisiae* in SGM (Supplementary Table S11). These compounds, that in moderate concentrations positively influence aroma complexity, could have a negative impact in higher concentrations, masking some other minor varietal aroma compounds (Belda et al., 2017). *C. fabianii* showed a similar production of higher alcohols than *S. cerevisiae,* but it is

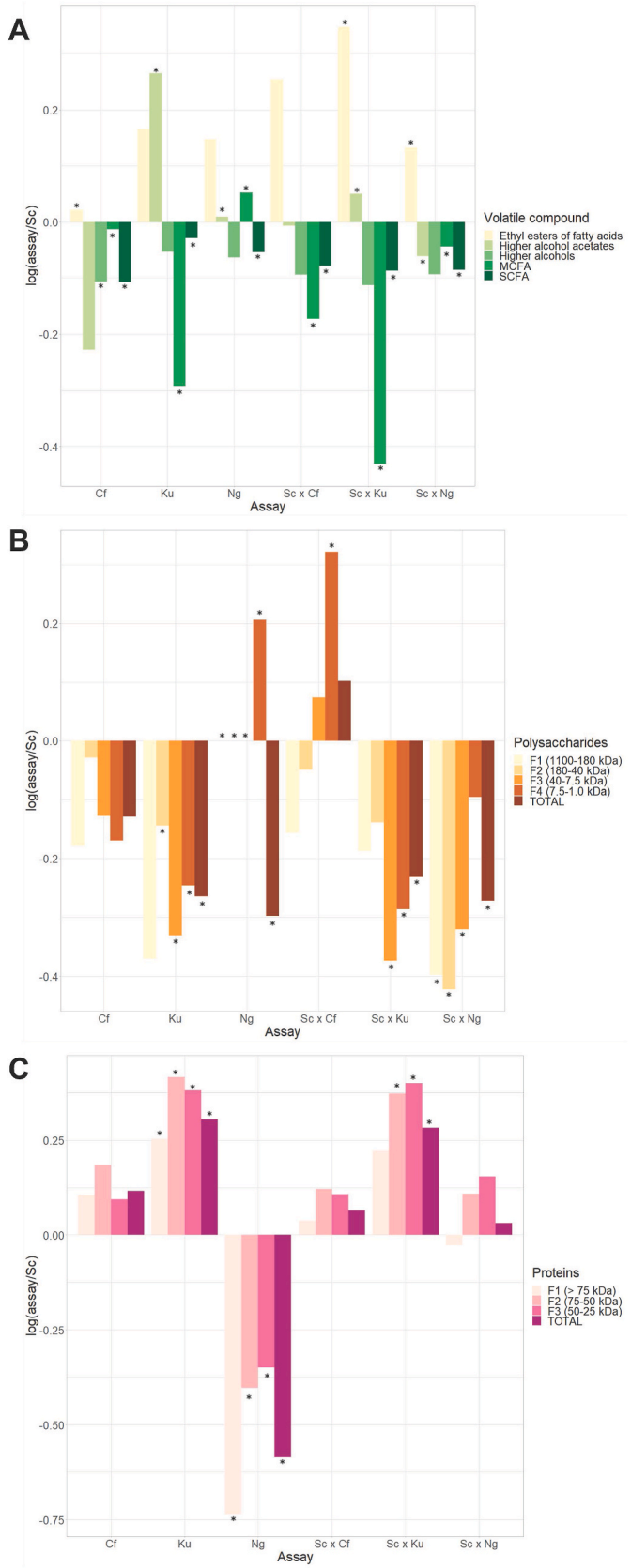

Fig. 4. Polysaccharides (A), proteins (B), and volatile compounds (C) analysed at the end of the different fermentation assays performed in SGM, in comparison with the Sc assays (represented as log10 (assay/Sc)). Equivalent results from the assays in natural grape (var. *Verdejo*) must are shown in Supplementary Figure S4.

remarkable the production of 3-methyl-butanol (isoamyl alcohol) in the mixed inoculation ScxCf assays in SGM (Supplementary Table S13), a molecule which has been previously describe to participate in cell-cell communication (i.e. *quorum* sensing) in yeasts (Hogan, 2006); however, this effect was not observed in the more complex environment of natural grape must fermentations (Supplementary Table S12). In the case of *N. globosa*, although showing a low fermentation capacity (it was able to consume, on average, a 10% of the fermentable sugars, and a 40% of PAN in the SGM (Table 1)), there is a notable formation of higher alcohols (Fig. 4C), which could be produced either by catabolic process (Ehrlich pathway) or anabolically (consumption of glucose/fructose) (Ribéreau-Gayon et al., 2006), and the highest production of higher ethanol acetates (e.g. isobutyl acetate) in monoculture. However, the production of higher alcohol acetates was dramatically reduced in the co-inoculation assay with *S. cerevisiae* (ScxNg).

Our results highlight the importance of keeping the research on the impact of biotic factors (i.e. inter-species interactions) in fermentation performance by surveying the whole potential diversity of wine yeast species. Originally, most non-*Saccharomyces* yeasts were considered as spoilage yeasts, then, in the last decade a bunch of papers have explored their deliberate use to improve different aspects of wine quality; now it is time to promote research on yeast interaction patterns, both at the level of molecular mechanisms and from the perspective of community ecology. We also conclude that assaying the growth phenotype of yeasts in a wide panel of wine-related conditions gives important information about the environmental preferences and the potential metabolic niches to be exploited by wine-releated yeasts, and this may help to guide further hypothesis on the mechanism of action of inter-species interactions. In our work, although we did not detect an important contribution of the less fermentative yeasts studied (*N. globosa* and *C. fabianii*), we have found a strong negative interaction of *K. unispora* with *S. cerevisiae*. Since we did not detect the production of killer toxins from *K. unispora,* we hypothesise that its faster growth kinetics accompanied by a higher nitrogen consumption rate and a higher production of acetic acid during the early stage of the wine fermentation can account for the ability of *K. unispora* DN201 to cause stuck fermentations, both in fermentations with inoculated *S. cerevisiae* AG012 and with native populations. Comparing our results with the few data available in the literature, which did not detect an inhibitory effect of other *Kazachstania* strains on the fermentation performance of *S. cerevisiae* (Jood et al., 2017; Lin et al., 2022), should encourage further investigation on the inter- and intra-specific diversity of *Kazachstania* wine-releated yeasts, so we can confirm the biological metabolic features of *Kazachstania* strains causing stuck fermentations.

Declaration of competing interest

The authors do not have any conflict of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Accession codes for all DNA data used in this work are reported in the manuscript, and they are publicly available

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104189.

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