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Research Article

Growth Fitness of Indigenous Wine Yeasts in Grape Musts from Different *Vitis* Species

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Abstract

Yeast communities associated with *Vitis vinifera* L. niches (i.e. grapes and fermenting grape musts) have been widely characterized. Less is known, however, about yeast communities present in other non-*vinifera Vitis* ecosystems. Moreover, there are no studies concerning eventual must-specific growth fitness of indigenous wine yeast species. In this work, we have characterized the potential must-specific growth fitness of ten different indigenous wine yeast species (i.e. *C. azymoides*, *C. californica*, *H. uvarum*, *H. vineae*, *I. hanoiensis*, *M. pulcherrima*, *P. cecembensis*, *T. delbrueckii*, *S. bacillaris* and *S. cerevisiae*). All the analyzed strains were isolated from spontaneously fermenting musts of *V. vinifera* L. (cv. Malbec) and/or *V. labrusca* L. (cv. Isabella) grapes harvested from vineyards in a shared *terroir*. Yeast identification was performed using standard ITS-rDNA RFLPs and/or microsatellite genotyping. Growth fitness of selected yeast species and strains, on Malbec and Isabella pasteurized grape must media, was studied by measuring lag phases (i.e. Lag Time) and maximum growth rates (i.e. μmax). Results showed that rare yeast species isolated from the Isabella ecosystem (i.e. *P. cecembensis* and *I. hanoiensis*) have better growth parameters when growing in Isabella grape must. The growth parameters of other wine yeast species, isolated from Malbec and Isabella ecosystems, did not show any clear associations with their musts of origin. Our findings suggest that the presence of two rare yeast species in Isabella could result from their growth advantage to this *Vitis* ecosystem. It is possible that yeast communities assembled in alternative grape musts result from the growth fitness of yeast species and strains to each specific *Vitis* species. Non-conventional *Vitis* ecosystems may constitute a reservoir of unique yeast strains valuable in the winemaking industry.

Keywords: *Vitis vinifera* L.; *Vitis labrusca* L.; Grape Must; Yeast; Non-*Saccharomyces; Saccharomyces cerevisiae*; Microbial Fitness

Introduction

Spontaneously fermenting grape musts are complex biological and chemical ecosystems where a diverse community of microorganisms (i.e. yeast, bacteria and filamentous fungi) contributes to the final wine chemistry [1]. A rich diversity of non-*Saccharomyces* and *S. cerevisiae* wine yeast species predominate during alcoholic fermentations [2,3]. These dynamic microbiotas produce a large variety of secondary metabolites that defines the organoleptic profile of beverages [4]. Because of its scientific and industrial relevance, the study of the indigenous microbial communities in grapes and fermenting grape musts ecosystems constitutes a major research area in oenology [2,5-8].

The identity and relative abundance of the various yeast species found at early stages of must fermentation appear to be

determined by biotic (e.g. general microbiota, killer factors, grape variety, etc.) and abiotic (e.g. pH, temperature, osmotic pressure, ethanol, total nitrogen, SO_2, etc.) factors [9-11]. A common pattern of development of certain wine yeast species has been recognized in spontaneously fermenting musts from *Vitis vinifera* L. grapes, being non-*Saccharomyces* the most common yeast species at initial stages [2,5,12-14] and *S. cerevisiae* the dominant species at the middle and final stages of fermentation [3].

Extensive research has been conducted on the complexity and fermentation-driven dynamics of yeast communities in *V. vinifera* L. ecosystems [2,4]. However, only a few studies have examined the yeast communities in non-*vinifera Vitis* ecosystems [15]. These non-conventional *Vitis* ecosystems may harbor a rich diversity of yeast species and strains with unique metabolic traits [13,14]. The diversity of yeast communities in grapes and grape musts from *Vitis labrusca* L. cultivars and its hybrids has been studied in vineyards from Brazil [16,17], the Azores Archipelago (Portugal) [18] and Argentina [13,14]. These studies highlighted the remarkable diversity of non-*Saccharomyces* yeast species in these particular non-conventional *Vitis* ecosystems, also suggesting the existence of specific *Vitis*-yeast species associations [13,14].

In this work, we characterized fitness parameters of indigenous yeasts isolated from two different *Vitis* ecosystems. Yeast growth studies were performed using musts from Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) grapes harvested from neighboring vineyards. This experimental design allowed us to explore the growth fitness of indigenous wine yeast strains in grape musts from different neighbor *Vitis* ecosystems.

Materials and Methods

Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) grape musts

Malbec (*Vitis vinifera* L.) and Isabella (*Vitis labrusca* L.) grapes were harvested at their optimal ripeness stages from several vineyards in Colonia Caroya, located at 31º02'00"S/64º05'36"O and 491 meters above sea level, in the province of Córdoba, Argentina, during the 2018 vintage. The region has an annual rainfall of 765 mm and a mean temperature of 15.8ºC. Separate must samples were taken immediately after grape crushing from tanks located in a room of a local winery not previously used for winemaking. Grape musts were transported under refrigeration to the laboratory, and 1 l of each must was filtered and centrifuged at 3000 rpm for 10 min at room temperature. Supernatants were pasteurized in 180 ml aliquots in a water bath (70ºC, 30 min) and stored at -20°C for growth analyses. Physicochemical analyses of the musts (i.e. °Brix, α amino, ammonium, yeast assimilable nitrogen –YAN–, pH, total acidity, volatile acidity and density) were performed following standard oenological procedures [14].

Indigenous yeast strains

Yeast strains used in this study were isolated from spontaneously fermenting musts of *V. vinifera* L. and *V. labrusca* L. grapes, as previously described [13,14]. Additionally, the commercial *Saccharomyces cerevisiae* yeast strain Lalvin EC1118 (Lallemand, Canada) was included as a control. Strains used in this study are indicated in table 1. Yeasts were previously identified to species level by PCR-RFLP (Table 2) and/or DNA sequencing of their 5.8-ITS (*Internal Transcribed Spacer*) rDNA regions, using ITS1 and ITS4 primers [13,14]. 5.8-ITS sequences were deposited in the NCBI GenBank database under the accession numbers *KY693700* (*Candida azymoides*: IT0-016), *KY693709* (*Candida californica*: IT2-010), *VG734839* (*Hanseniaspora uvarum*: IT117-013), *VG734841* (*Hanseniaspora uvarum*: MT017-035), *KY693711* (*Hanseniaspora vineae*: IT2-021), *KY693701* (*Issatckenkia hanoiensis*: IT0-025), *KY693704* (*Pichia cecembensis*: IT0-042), *MG734849* (*Starmerella bacillaris*: MT017-001), *KY693706* (*S. bacillaris*: IT1-033), *MG734853* (*S. cerevisiae*: MT217-023), *MG734858* (*S. cerevisiae*: IT217-022), and *KY693707* (*T. delbrueckii*: IT1-039). Furthermore, *S. cerevisiae* and *S. bacillaris* isolates were characterized to strain level by microsatellite genotyping [19,20].

Yeast growth analyses in alternative grape musts

To assess yeast growth in alternative media, selected strains corresponding to ten different yeast species (Table 1), were cultivated in Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) grape musts as well as YPD [yeast extract 1.0% (w/v), peptone 2.0% (w/v), glucose 2.0%] media. Yeast strains were grown in 100-well honeycomb plates (Growth Curves, USA) filled with 200 µl of filtered and pasteurized grape must or sterile YPD media. Yeasts were pre-cultivated in YPD media for 16h at 25°C and inoculated into the grape musts or liquid YPD to a final concentration of 1 x 10^6 cells/ml. Growth was monitored by OD_{600} measurements for 48 hours at 25°C, with constant agitation, using a microplate spectrophotometer (Bioscreen C©). The well position on the microplate was randomized and four replicates were run for each strain and growth media condition.

Yeast species	Must[1]	Year[2]	Isolate
S. cerevisiae	Commercial	-	EC1118
	Malbec	2017	MT217-023
	Isabella	2016	IT1217-022
S. bacillaris	Malbec	2017	MT017-001
	Isabella	2016	IT1-033
T. delbrueckii	Malbec	2017	MT017-059
	Isabella	2016	IT1-039
H. uvarum	Malbec	2017	MT017-035
	Isabella	2017	IT117-013
H. vineae	Malbec	2017	MT017-056
	Isabella	2016	IT2-021
C. azymoides	Malbec	2017	MT017-061
	Isabella	2016	IT0-016
M. pulcherrima	Malbec	2017	MT117-022
C. californica	Isabella	2016	IT2-010
I. hanoiensis	Isabella	2016	IT0-025
P. cecembensis	Isabella	2016	IT0-042

Table 1: Analyzed yeast strains.

[1]: Spontaneously fermenting must; [2]: Year of isolation.

Yeast species	PCR product (size; bp)	RFLP[1] pattern	
		CfoI	HinfI
S. cerevisiae	850	388 + 359 + 141	373 + 375 + 120
S. bacillaris	460	56 + 103 + 105 + 196	225 + 235
T. delbrueckii	800	359 + 243 + 159 + 108	473 + 400
H. uvarum	770	359 + 340 + 116	376 + 210 + 178
H. vineae	754	270 + 156 + 143 + 93	389 + 373
C. azymoides	480	213 + 206 + 80	257 + 206
M. pulcherrima	404	233 + 106 + 93	222 + 196
C. californica	482	238 + 109 + 71	269 + 225
I. hanoiensis	472	137 + 98 + 86 + 66	272 + 228
P. cecembensis	496	256 + 116 + 64 + 47	277 + 129

Table 2: DNA digestion patterns of ITS-5.8S regions of various yeast species.

[1]: Restriction fragment length polymorphism (i.e. bp).

Data analysis of growth measurements

Data from the microplate reader were transformed with the polynomial curve y = -0.0018*x3 + 0.1464*x2 + 0.7757*x + 0.0386 to correct the non-linearity of the optical recording at higher cell densities as previously reported [21]. Growth kinetic data were fitted using the Richards flexible inflection point model implemented by the fit growth model function, R package growth rates. This model allows the estimation of the maximal growth rate (μmax). A second parameter, Lag Time, was manually computed from raw data by considering the time necessary to reach twice the OD_{600} of the inoculums [19]. The following full linear model was applied for estimating the effects of the must of isolation and the growth media and their possible interactions: Lm1: Yik = m + Im i + Gm k + (Im:Gm) ik + E ijk, where Y is the value of the trait (μmax and Lag Time), m is the overall mean, for j: Im (isolation must, i = 1 to 2), and Gm (growth media, k = 1 to 3), and E is the residual error. Homoscedasticity of the ANOVA was tested by Levene Test function and visual inspection of dispersion diagrams (car package), while the normal distribution of models' residuals was estimated by visual inspection (qq plot). Comparisons between treatments were performed by Tukey test (Infostat; Universidad Nacional de Córdoba).

Results

Indigenous yeasts in spontaneously fermenting musts of grapes harvested in neighbor *Vitis vinifera* L. and *Vitis labrusca* L. ecosystems

Remarkable differences have been recognized in the diversity and identity of non-*Saccharomyces* species isolated from *V. vinifera* L. and *V.* non-*vinifera* ecosystems (See Raymond Eder and Rosa, 2019). In this work we characterized a collection of indigenous yeast isolates from spontaneously fermenting musts of Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) grapes harvested from neighbor vineyards (see Materials and Methods section). Table 2 shows the ITS-rDNA RFLP patterns of some rare wine yeasts, used to establish species identity. ITS-rDNA PCR products were also sequenced to further verify the identity of the species analyzed in this work (See materials and methods section).

Among the isolates shown in table 1, some yeast species rarely isolated from *V. vinifera* L. fermenting grape musts can be identified (i.e. *Candida azymoides*, *Candida californica*, *Issatchenkia hanoiensis and Pichia cecembensis*). A comparison of the large yeast

biodiversity present at early stages of the Malbec and Isabella spontaneously fermenting grape musts [14] is shown in figure 1. Previous microsatellite loci genotyping studies of a large number of strains from a representative non-*Saccharomyces* yeast species (i.e. *S. bacillaris*) and *S. cerevisiae*, isolated from Malbec and Isabella ecosystems [19,22], also revealed a great biodiversity of strains of these yeast species. Taken together, these results highlight the remarkable biodiversity and presence of extraordinary wine yeasts in the non-*vinifera Vitis* ecosystem Isabella.

Figure 1: Diversity of non-*Saccharomyces* species isolated from spontaneously fermenting must of *V. vinifera* L. and *V. labrusca* L. grapes harvested in a shared terroir. Relative contribution of non-*Saccharomyces* yeast species representing more than 1% of the isolates from spontaneously fermenting *V. labrusca* L. (A) and *V. vinifera* L. (B) grape musts (vintage 2017). Numbers in parentheses indicate percentages. Non-*Saccharomyces* species are: *Ca* (*Candida azymoides*), *Cap* (*Candida apicola*), *Cc* (*Candida californica*), *Ch* (*Candida hellenica*), *Hu* (*Hanseniaspora uvarum*), *Hv* (*Hanseniaspora vineae*), *Lt* (*Lachancea thermotolerans*), *Mp* (*Metschnikowia pulcherrima*), *Pc* (*Pichia cecembensis*), *Pk* (*Pichia kluyveri*), *Pku* (*Pichia kudriavzevii*), *Pn* (*Pichia norvegensis*), *Po* (*Pichia occidentalis*), *Pt* (*Pichia terricola*), *Sb* (*Starmerella bacillaris*) and *Td* (*Torulaspora delbrueckii*).

Growth fitness of indigenous yeasts in Malbec and Isabella grape musts

The remarkable yeast biodiversity observed in neighboring *Vitis* ecosystems (i.e. *V. vinifera* L. and *V. labrusca* L.) from the same geographic region (Table 1 and figure 1) suggested that specific traits of different *Vitis* species could contribute to structure their associated, specific yeast communities (i.e. yeast species and/or strains) [23].

To address the above mentioned hypotheses, in this work we explored the growth phenotypes of a subset of 16 indigenous yeast strains corresponding to ten species isolated from *Malbec* and *Isabella* fermenting grape musts (Table 1). We hypothesized that yeast strains isolated from a specific *Vitis* grape must could have a competitive advantage when grown in their must ecosystem of origin. In these studies, growth fitness in pasteurized Malbec and Isabella grape musts was assessed for each strain (See materials and methods section). The fitted OD_{600} growth data (Figure 2) allowed the estimation of the maximum growth rate (i.e. μmax) and the Lag phase (i.e. Lag Time) was also calculated using the growth raw data (Figure 3). The effect of the original must of isolation and the tested must for growth was estimated by two-way ANOVA (model Lm1). Table 3 shows that the growth media significantly impacted the two parameters (i.e. μmax and Lag Time) in most of the strains tested, explaining much of their variance.

Figure 2: Fitting of growth kinetic data. Example of the analysis of *S. bacillaris* IT1-033 growth in Malbec grape must. (A) Raw OD_{600} growth data. (B) Fitted OD_{600} data using the Richards flexible inflection point model. The fit of the model is shown as a continuous blue line.

Tukey's analyses of the data (Table 4) showed that *I. hanoiensis*, originally isolated from the Isabella ecosystem, showed better growth parameters (i.e. shorter Lag time and higher μmax) when grown in Isabella grape must. Interestingly, although the growth must (i.e. Malbec or Isabella) did not impart a significant difference in the Lag phase of *P. cecembensis*, a remarkable higher maximum

Figure 3: Growth of indigenous yeast species in Malbec, Isabella and YPD media. (A) Mean of the Lag Times (in hours) of each strain in the alternative Malbec (M), Isabella (I) and YPD (Y) growth media. (B) Mean of the μmax of each strain in the alternative Malbec (M), Isabella (I) and YPD (Y) growth media. Ca I (*C. azymoides,* Isabella isolate), Ca M (*C. azymoides,* Malbec isolate), Cc I (*C. californica,* Isabella isolate), Hu I (*H. uvarum,* Isabella isolate), Hu M (*H. uvarum,* Malbec isolate), Hv I (*H. vineae,* Isabella isolate), Hv M (*H. vineae,* Malbec isolate), Ih I (*I. hanoiensis,* Isabella isolate), Mp M (*M. pulcherrima,* Malbec isolate), Pc I (*P. cecembensis,* Isabella isolate), Sb I (*S. bacillaris,* Isabella isolate), Sb M (*S. bacillaris,* Malbec isolate), Sc I (*S. cerevisiae,* Isabella isolate), Sc M (*S. cerevisiae,* Malbec isolate), Td I (*T. delbrueckii,* Isabella isolate), Td M (*T. delbrueckii,* Malbec isolate) and Sc C (*S. cerevisiae,* Commercial isolate).

Yeast species	Isolation must		Growth media		Im*Gm[1]		
	Effect	p-value	Effect	p-value	Effect	p-value	
S. cerevisiae	na	ns	16.7	.	52.4	***	Lag Time
S. bacillaris	33.9	***	16.9	*	37.3	***	
T. delbrueckii	50.9	***	21.6	*	11.8	.	
H. uvarum	na	ns	na	ns	na	ns	
H. vineae	62.5	***	na	ns	na	ns	
C. azymoides	48.8	***	9.0	.	13.5	*	
M. pulcherrima	na	na	82.6	.	na	na	
C. californica	na	na	75.4	.	na	na	
I. hanoiensis	na	na	58.3	.	na	na	
P. cecembensis	na	na	82.4	.	na	na	
S. cerevisiae	10.1	***	12.3	***	59.8	***	μmax
S. bacillaris	na	ns	78.6	***	na	ns	
T. delbrueckii	13.7	.	na	ns	44.1	*	
H. uvarum	na	ns	58.8	***	na	ns	
H. vineae	0.7	.	97.8	***	na	ns	
C. azymoides	na	ns	91.1	***	na	ns	
M. pulcherrima	na	na	98.2	***	na	na	
C. californica	na	na	56.5	.	na	na	
I. hanoiensis	na	na	93.8	***	na	na	
P. cecembensis	na	na	93.9	***	na	na	

[1]Isolation must (Im) and Growth media (Gm) interactions; significance levels: '***' <0.0001; '**'<0.001; '*' <0.01; '.' <0.05; ns: not significant; na: not applicable.

Table 3: Analyses of variance of isolation must and growth media interactions for Lag time and μmax.

growth rate for this strain was observed in Isabella must (Table 4). Growth parameters of other yeast species, either rarely or ubiquitously recognized in Malbec and/or Isabella ecosystems, were significantly affected by the interactions between the two analyzed factors (Table 3). For example, *C. californica*, a yeast species specifically associated with the Isabella ecosystem, showed a better growth parameter (i.e. lower Lag phase) in Malbec must. For *C. azymoides*, the strain isolated from Malbec revealed a fastest growth in the Isabella must, while the Isabella isolate did not show differences in the Lag phases in both musts. Finally, the Malbec isolates from *T. delbrueckii* and *S. bacillaris* showed lower Lag Times in both grape musts than the Isabella isolates. Other yeast species (i.e. *H. uvarum* and *H. vineae*) did not show significant differences in the growth parameters for the interactions between the two factors analyzed (Table 3).

Physicochemical analyses of the Malbec and Isabella grape musts

Results from the physicochemical analyses of the Malbec and Isabella grape musts are shown in table 5. As it was previously

Yeast species	Isolation must	Growth media[1]					
		Lag Time			μmax		
		M	I	Y	M	I	Y
S. cerevisiae	Commercial	e	abcd	a	def	def	c
	Malbec	bcde	de	abc	cd	ef	a
	Isabella	ab	cde	abcde	cde	b	f
S. bacillaris	Malbec	a	a	ab	na		
	Isabella	c	bc	a			
T. delbrueckii	Malbec	a	a	a	ab	ab	ab
	Isabella	b	b	a	b	ab	ab
C. azymoides	Malbec	b	a	a	na		
	Isabella	bc	c	bc			
M. pulcherrima	Malbec	b	b	a	a	c	b
C. californica	Isabella	a	b	b	ab	b	a
I. hanoiensis	Isabella	b	a	ab	a	b	b
P. cecembensis	Isabella	b	b	a	a	c	b

[1]M, Malbec grape must; I, Isabella grape must; Y, YPD media. Tukey analyses for strains isolated from only one must are shown in gray. Values with a common letter are not significantly different ($p > 0.05$); mean values: a < f. na: not applicable.

Table 4: Tukey analyses of isolation must and growth media interactions for Lag time and μmax.

described [14], the initial content of reducing sugars (°Brix) was higher in the Malbec must than in the Isabella grape must. Furthermore, the Malbec and Isabella pH values were consistent with previous analyses [13,14]. The greatest differences were observed among the content of nitrogen compounds in the musts (i.e. α Amino, Ammonium, and Yeast Assimilable Nitrogen –YAN–), which were higher for the Isabella musts than for the Malbec musts analyzed.

Discussion and Conclusion

There is extensive research on the characterization of the microbiological communities associated with *Vitis vinifera* L. grapes and musts that contribute to the fermentation process. Grape varieties themselves could condition the assembly and dynamics of the microbial population during spontaneous fermentation [24-26]. A more diverse non-*Saccharomyces* and *S. cerevisiae* strain population has been recognized in vineyards having several grape varieties than vineyards cultivating only one grape variety [25,26]. This observation suggests that vineyards harboring multiple *Vitis* species may show a more diverse yeast community as compared to vineyards with a single *Vitis* species. Thus, specific structural and/or general physicochemical factors of each grape varietal may influence the structure and fitness of their specific grape yeast microbiota [27]. Interestingly, non-*vinifera Vitis* species, a poorly characterized microbial niche, harbor wine yeast species rarely recognized in the conventional *V. vinifera* L. ecosystem [13,14].

In this study, we hypothesized that specific growth fitness of indigenous strains could be the result of a growth adaptive advantage to their *Vitis* ecosystem of origin. To explore this idea, we characterized the growth fitness of indigenous yeast species,

Must	°Brix	α Amino (mg/l)	NH_4^+ (mg/l)	YAN (mg/l)	pH	Total acidity (g/l)	Volatile acidity (g/l)	Density (g/ml)
Malbec	21.4	45.3	37.9	76.38	3.74	2.5	0.18	1.089
Isabella	18.4	104.9	62.5	156.18	3.56	3.1	0.17	1.080

Table 5: Chemical analyses of Malbec and Isabella grape musts.

isolated from *V. vinifera* L. and *V. labrusca* L. fermenting grape musts, on grape must from these alternative *Vitis* ecosystems. Relevant growth parameters of sixteen indigenous yeast isolates, corresponding to ten different wine yeast species, were determined in pasteurized Malbec and Isabella grape musts. As a control of yeast growth, all the studies were also performed in a nitrogen rich, limited in reducing sugars (i.e. glucose), medium (i.e. YPD). This medium served as a reference for the growth of all the yeast strains tested. OD_{600} growth data were fitted using the Richards flexible inflection point model to determine the maximum growth rate (i.e. μmax) parameter. Lag Times were also estimated by considering the time necessary to reach twice the OD_{600} of the inoculums.

Our studies showed that *I. hanoiensis* and *P. cecembensis* isolates, rare yeast species preferentially associated with the Isabella ecosystem [13], have higher μmax values when growing in Isabella grape must. From this limited evidence, it is tempting to speculate that the presence of some rare yeast species in the Isabella ecosystem could result from their selective growth advantage to this *Vitis* species. The evaluation of the growth parameters of *C. azymoides*, *C. californica*, *H. uvarum*, *H. vineae*, *M. pulcherrima*, *T. delbrueckii*, *S. bacillaris* and *S. cerevisiae* indigenous strains, on the other hand, did not show a defined fitness preference for their corresponding *Vitis* ecosystem of isolation. Further studies are required to identify the factors of the Isabella grape musts specifically favoring the growth of *I. hanoiensis* and *P. cecembensis* yeast species.

Author Contributions

MLRE and ALR equally contributed to the conception, drafting, revising and final approval of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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