UDC 663.14:663.251
ISSN 1330-9862

scientific note

Dynamics of Yeast Populations during the Early Stages of Natural Fermentations for the Production of *Brunello di Montalcino* Wines

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Received: September 9, 1997
Accepted: November 11, 1998

Summary

The growth behaviour of the individual yeast species dominating the early stages of natural fermentations of grape musts for the production of quality Brunello di Montalcino wines in the course of three consecutive vintages was quantitatively determined, under commercial conditions in two different cellars, and following the time course of fermentation parameters. Freshly extracted grape juices, sulfured to initial concentrations between 40 and 100 mg of total SO_2 per liter, contained 10^3 to 10^6 yeast cells per mL, depending on the vintage. Kloeckera apiculata, Metschnikowia pulcherrima and, occasionally, Candida stellata and Candida valida were the dominant species. Saccharomyces cerevisiae was present in much lower numbers than the non-Saccharomyces species. Independently from the initial SO_2 concentration, a significant growth of non-Saccharomyces yeasts was generally observed before the onset of the vigorous fermentation phase. The kinetic characteristics of this growth were found to vary with the fermentation as well as with the yeast species. K. apiculata remained the species with the highest specific growth rate and yield, reaching maximum density of about 10^8 cells per mL. After growing to maximum density, the non-Saccharomyces yeasts rapidly lost their viability. The decline phase of K. apiculata started once Sacch. cerevisiae became the dominant yeast, rather than once ethanol and temperature reached values known to inhibit growth of apiculate yeasts. Sacch. cerevisiae showed only slight differences in its growth behaviour, with maximum specific growth rates quite similar to those of K. apiculata. The rate of ethanol production during the vigorous fermentation phase seemed to be affected by the extent to which the density of non-Saccharomyces yeasts grew.

Keywords: wine microbiology, *non-Saccharomyces yeasts*, natural wine fermentation

Introduction

Natural fermentation of grape juice is known to involve various species of yeasts that originate from grapes and winery equipments and that exhibit a sequential development during vinification (*1–4*). Non-*Saccharomyces* yeasts generally predominate in the early stages of fermentation, whereas *Saccharomyces cerevisiae* becomes the dominant yeast only after the first three to five days. Growth of non-*Saccharomyces* species to maximum density of 10^6 to 10^8 cells per mL may occur and possibly affect both the chemical composition of the wine and the growth kinetics as well as the biochemical activities of *Sacch. cerevisiae* (*5–7*). However, despite the important role that non-*Saccharomyces* density may play in determining the sensory profiles of wines, only very few quantitative data are available on the growth behaviour of individual yeast species during grape must fermentation under commercial conditions (*5,6,8–10*). In this study, the growth behaviour of the individual yeast species occurring in the early stages of natural vinification of quality *Brunello di Montalcino* wines in the course of three consecutive vintages was quantitatively determined, under commercial conditions in two different cellars, and following the time course of the main fermentation parameters.

Materials and Methods

Wine fermentations

Grapes of Sangiovese variety for *Brunello di Montalcino* wine production were harvested during the 1994 to 1996 vintages from two neighbouring vineyards of the «Case Basse» winery in Montalcino, Italy. Vinifications were carried out without inoculation in two different cellars of the same winery, referred to as A and B, in wooden tanks of 15,200 and 12,200 liter capacity, respectively. In the 1994, 1995 and 1996 vintages, the filling percentages of the tanks were 73, 80 and 40 for tank A and 80, 58 and 54 for tank B. In Table 1, the chemical characteristics of the musts as well as the initial concentrations of added sulfur dioxide are reported.

Table 1. Chemical characteristics of the freshly extracted grape musts

Parameters	Cellar A			Cellar B		
	1994	1995	1996	1994	1995	1996
γ(glucose) / g L^{-1}	104	118	121	95	120	117
γ(fructose) / g L^{-1}	135	130	132	122	131	124
Total acidity as γ(tartaric acid) / g L^{-1}	7.4	7.5	7.0	7.3	7.2	7.5
pH	3.41	3.40	3.2	3.40	3.25	3.16
γ(total SO_2) / mg L^{-1}	36	102	58	36	77	58
γ(free SO_2 / mg L^{-1}	8	51	10	8	38	10

Enumeration, isolation and identification of yeasts

Wine samples were taken, soon after »*remontages*«, from the middle of the tanks and immediately transferred to laboratories for analysis. Yeast enumeration was obtained by surface spreading 0.1 mL samples of wine (diluted if necessary) onto plates of WL nutrient agar and lysine agar (*8,11*). To reveal the presence of *Sacch. cerevisiae* 10% ethanol and hydrogen sulfite (metabisulfite), 150 mg L^{-1}, were added to WL nutrient agar, as suggested by Kish *et al.* (*12*). Representative colonies, developed after incubation of the plates at 28 °C, were isolated and then identified according to the procedures and keys of Kreger-van Rij (*13*) and Barnett *et al.* (*14*). *Saccharomyces cerevisiae* isolates were identified as suggested by Vaughan-Martini and Martini (*15*). The results were analysed by Yeast Identification PC Program, version 4 (*16*).

Chemical analysis

Sugar and ethanol concentrations were determined by a Beckman 340 liquid chromatograph equipped with refractive index (Beckman 156) and a Biorad Aminex HPX-87H column (300 mm × 7.8 mm I.D.). The column was operated at 65 °C with a mobile phase of 6.3 mM H_2SO_4 (flow rate 0.6 mL min^{-1}). For quantitative determinations, peak areas were compared to a calibration curve. Total and free SO_2 concentrations and total acidity were determined according to the official methods for wine analysis (*17*).

Results

The growth behaviour of individual yeast species as well as the time course of main fermentation parameters during the early stages of different vinifications are shown in Figs. 1–3. Depending on the vintage, density of non-*Saccharomyces* yeasts in freshly extracted grape musts ranged from about 10^3 to 10^6 CFU mL^{-1}, dominant yeasts always belonging to few species: *Kloeckera apiculata, Metschnikowia pulcherrima* and, occasionally, *Candida stellata* and *Candida valida. Saccharomyces cerevisiae* was never isolated from fresh musts by the usual plating media, so that its initial presence should have been always in much lower numbers than the non-*Saccharomyces* species. Indeed, when the medium containing both ethanol and hydrogen sulfite (metabisulfite) was additionally used (1996 vintage), *Sacch. cerevisiae* was actually isolated from fresh musts, but it accounted for only very few CFU mL^{-1} against a non-*Saccharomyces* density of approximately 10^6 CFU mL^{-1} (Fig. 3).

In the vinifications containing initial concentrations of total SO_2 higher than 50 mg L^{-1} (Table 1), during the first 24 to 48 hours of fermentation, non-*Saccharomyces* yeasts generally reduced their initial numbers. However, the extent to which individual yeast species died away varied with the vinification, both within the same vintage and among different vintages (Figs. 2 and 3). In any case, independently from the initial SO_2 concentration, a significant growth of non-*Saccharomyces* yeasts was generally observed before the onset of the vigorous fermentation phase. The kinetic characteristics of this growth were found to vary with the fermentation as well as with the yeast species. Depending on the fermentation, the population of *Kloeckera apiculata*, that dominated the early stages of almost all fermentations, increased by 2 to 4 log units, reaching maximum values of more than 10^7 CFU mL^{-1}. This apiculate yeast was the non-*Saccharomyces* species showing the highest maximum specific growth rates, with slight differences among the fermentations (Table 2). *Metschnikowia pulcherrima* and *Candida stellata* showed more variable growth kinetics than *K. apiculata*, in spite of the very similar conditions of temperature and ethanol that occurred in the different fermentations when the maximum specific growth rates were attained. Maximum density of *M. pulcherrima* never exceeded 10^5 CFU mL^{-1}, whereas *C. stellata*, in both 1996 fermentations, reached approximately $5 \cdot 10^6$ CFU mL^{-1}. After growing to maximum density, the non-*Saccharomyces* yeasts rapidly lost their viability. *M. pulcherrima* was the first species to die off, whereas *C. stellata* showed the longest persistence in fermenting musts, being able to maintain a sustained presence at ethanol volume fraction of about 9% and at temperatures of about 35 °C, in 1996 and 1995 vintages. As for *K. apiculata*, the pattern of its stationary and decline phases varied with the vinification. Indeed, in the 1995 vintage, the decline phase of *K. apiculata* started immediately after maximum density was reached, in spite of the very low values of ethanol concentration and temperature occurring in the tanks. By contrast, in the 1996 vintage, when *K. apiculata* grew to maximum density of about $5 \cdot 10^7$ CFU mL^{-1}, the species did not survive as long as *C. stellata* but showed a high degree of persistence, remaining at

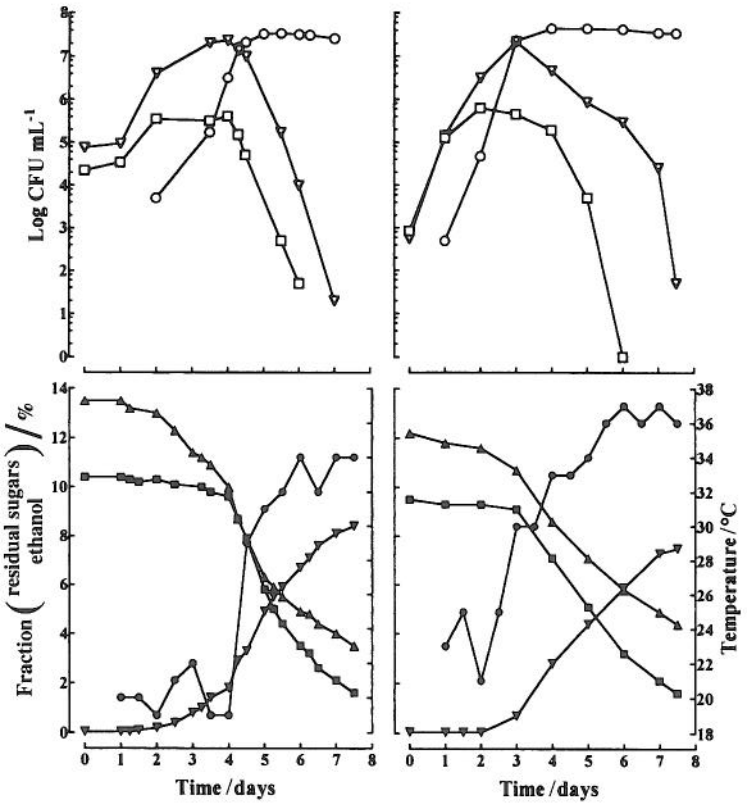

Fig. 1. Growth of yeast species and time course of main fermentation parameters during the 1994 vinification in cellars A (left) and B (right). Symbols: (▽) *Kloeckera apiculata*,(□) *Metschnikowia pulcherrima*, (○) *Saccharomyces cerevisiae*, (▲) fructose (mass fraction); (■) glucose (mass fraction); (▼) ethanol (volume fraction); (●) temperature.

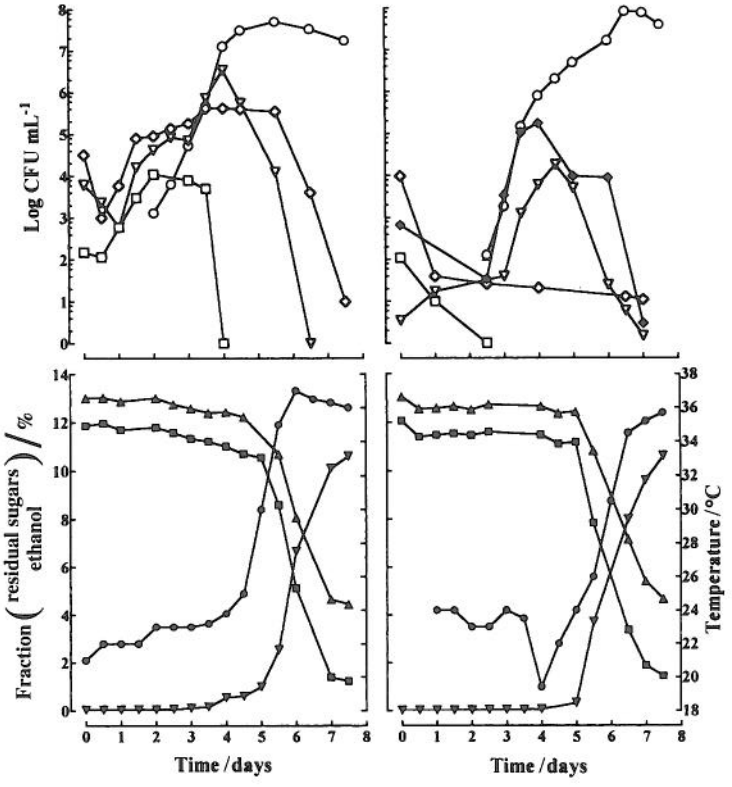

Fig. 2. Growth of yeast species and time course of main fermentation parameters during the 1995 vinification in cellars A (left) and B (right). Symbols: (▽) *Kloeckera apiculata*, (□) *Metschnikowia pulcherrima*, (◊) *Candida stellata*, (◆) *Candida valida*, (○) *Saccharomyces cerevisiae*, (▲) fructose (mass fraction); (■) glucose (mass fraction); (▼) ethanol (volume fraction); (●) temperature.

densities of 10^6–10^7 CFU mL^{-1} in the presence of ethanol volume fraction of 8% and temperature of 28 °C. Independently from the growth behaviour of non-*Saccharomyces* yeasts, *Saccharomyces cerevisiae* showed growth kinetics quite similar among the fermentations, exhibiting maximum specific growth rates that did not differ very much from those of *K. apiculata* (Table 2).

Table 2. Maximum specific growth rates (h^{-1}) of yeast species during fermentations

Yeast species	Cellar A			Cellar B		
	1994	1995	1996	1994	1995	1996
K. apiculata	0.157 (24–48)*	0.275 (24–36)	0.155 (24–42)	0.231 (0–24)	0.287 (72–84)	0.258 (24–48)
M. pulcherrima	0.097 (24–48)	0.136 (12–24)	0.139 (24–42)	0.208 (0–24)	n.g.	0.050 (0–48)
C. stellata	n.p.	0.216 (24–36)	0.086 (42–66)	n.p.	n.g.	0.104 (48–72)
C. valida	n.p	n.p.	n.p.	n.p.	0.279 (72–84)	n.p.
I. terricola	n.p.	n.p.	0.113 (24–42)	n.p.	n.p.	0.062 (24–48)
Sacch. cerevisiae	0.252 (84–102)	0.274 (84–96)	0.273 (42–66)	0.258 (48–72)	0.364 (72–84)	0.307 (48–72)

* Time period (h) taken into consideration for growth rate calculation. n.g. = no growth, n.p. = not present

As for the effects of the non-*Saccharomyces* yeast growth on the fermentation process, it is worth mentioning that a higher fructose consumption occurred in the early stages of the 1994 and 1996 vinifications, where *K. apiculata* exhibited significant growth to density of more than 10^7 CFU mL^{-1}. Furthemore, during the vigorous phase of these fermentations, both maximum ethanol production (mL L^{-1} day^{-1}) and specific rate of ethanol production [mL $(10^9$ viable cells$)^{-1}$ day^{-1}] were significantly lower compared to the 1995 vinifications, characterized by maximum density of non-*Saccharomyces* yeasts well below 10^7 CFU mL^{-1} (Table 3).

Discussion

The growth behaviour of the few yeast species that dominated the early stages of the grape must fermentations outlined in this study was in good accordance with most observations reported in the literature (*5–10, 18*). Nevertheless, the quantitative data described above, dealing with natural fermentations for the production of quality wines over three consecutive vintages, provide a sound description of the yeast growth kinetics under commercial conditions. Furthermore, the data allow further comments and speculations.

Sulphur dioxide addition, to initial concentrations in the usual range of 50–100 mg L^{-1} (total), did not succeed in preventing the growth of indigenous non-*Saccha-*

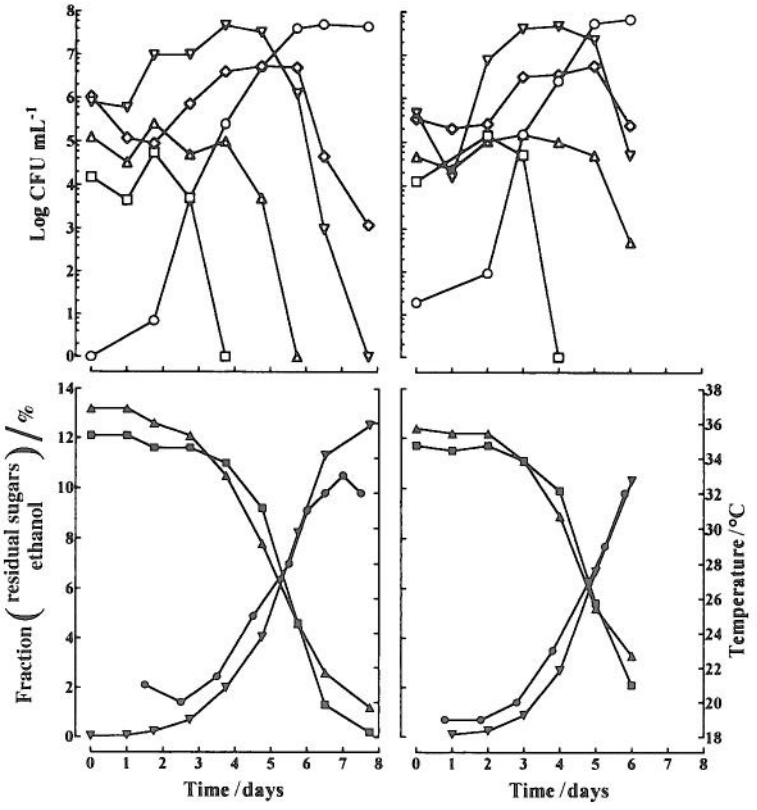

Fig. 3. Growth of yeast species and time course of main fermentation parameters during the 1996 vinification in cellars A (left) and B (right). At day 6.5, the content of the tank in the cellar B was transferred into the tank in the other cellar.
Symbols: (▽) *Kloeckera apiculata*, (□) *Metschnikowia pulcherrima*, (◇) *Candida stellata*, (△) *Issatchenkia terricola*, (○) *Saccharomyces cerevisiae*, the first two points were obtained from the medium WL with added sulphite and ethanol (▲) fructose (mass fraction); (■) glucose (mass fraction); (▼) ethanol (volume fraction); (●) temperature.

Table 3. Ethanol production and temperature range during the vigorous fermentation phase

Vinification	Maximum ethanol productivity mL L^{-1} day^{-1}	Specific rate of ethanol production mL (10^9 viable cells)$^{-1}$ day^{-1}	Temperature range (°C)
Cellar A			
1994	24 (96–144)*	0.7	29–34
1995	57 (120–144)	1.4	30–37
1996	41 (114–156)	0.9	26–32
Cellar B			
1994	17 (72–144)	0.4	30–37
1995	51 (120–156)	2.2	24–34
1996	38 (96–144)	0.5	24–32

* Time period (h) taken into consideration

romyces yeasts before the onset of vigorous fermentation, strengthening those questions that have been sometimes raised about the effectiveness of SO_2 controlling the growth of non-*Saccharomyces* yeasts (*4,6,19–21*). Moreover, if the differences in maximum specific growth rates of *K. apiculata* are considered (Table 2), no correlation can be found between the growth rate values and added SO_2 or pH or temperature of the musts. These findings also raise some doubts about the inhibitory effect that SO_2 is expected to exert on growth rates of non-*Saccharomyces* yeasts (*4,21*).

The maximum growth rates of *K. apiculata* did not differ very much from those of *Sacch. cerevisiae* (Table 2), but the growth profiles of the two species in the fermenting musts clearly show that the mean growth rates of the former species were not as high as for the latter. This experimental evidence raises new questions about the reasons for slowing down of the growth rate of *K. apiculata* at ethanol and temperature values far from being restrictive for its growth. The beginning of the decline phase for *K. apiculata* was always observed once *Sacch. cerevisiae* reached high cell density, becoming the dominant yeast, rather than concomitant with ethanol and/or temperature values known to affect *K. apiculata* survival (*7,22*). This phenomenon deserves further investigations to understand whether some kinds of interactions between *Sacch. cerevisiae* and the apiculate yeast arise during fermentation of grape juice. In this connection, growth of *K. apiculata* has been often reported to exert inhibitory effects on the growth of *Sacch. cerevisiae* (*23,24*). Indeed, on the basis of the growth kinetics exhibited by *Sacch. cerevisiae* in this study, and especially in those vinifications where *K. apiculata* reached density of about $5 \cdot 10^7$ CFU mL^{-1}, it seems that marked inhibitory effects of *K. apiculata* on *Sacch. cerevisiae* growth kinetics are to be excluded. By contrast, the significant growth of non-*Saccharomyces* yeasts to maximum densities of more than 10^7 CFU mL^{-1} seemed to exert a certain inhibitory effect on the ethanol production rates during the vigorous phase of fermentation.

As a concluding remark, it is to be noted that major differences in the patterns of yeast growth occurred among different vintages, rather than between the two cellars in the same vintage. These differences mainly involved non-*Saccharomyces* yeasts that, therefore, proved themselves a major cause of diversity in the ecology of these uninoculated commercial wine fermentations.

Acknowledgments

Mr. Gianfranco Soldera, the owner of the winery, is greatly acknowledged for the facilities and cooperation furnished. The authors also thank Massimo Anichini for his valuable technical assistance.

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Dinamika rasta stanica kvasca u ranim stadijima fermentacije pri proizvodnji vina *Brunello di Montalcino*

Sažetak

Kvantitativno je određen rast pojedinih sojeva kvasaca koji su prevladavali u ranim stadijima prirodne fermentacije grožđanog mošta pri proizvodnji kvalitetnih vina Brunello di Montalcino. Tijekom tri uzastopne berbe praćeni su fermentacijski pokazatelji, pri komercijalnim uvjetima, u dva različita podruma. Svježi sok od grožđa, sumporen do početne koncentracije između 40 i 100 mg ukupnog SO_2/L, sadržavao je 10^3–10^6 stanica kvasca/mL, ovisno o berbi. Dominantni sojevi bili su Kloeckera apiculata, Metschnikowia pulcherrima te katkad Candida stellata i Candida valida. Utvrđena je prisutnost puno većeg broja ne-Saccharomyces sojeva nego Saccharomyces cerevisiae. Neovisno o početnoj koncentraciji SO_2 opažen je znatan rast ne-Saccharomyces sojeva prije nastupa burne fermentacijske faze. Kinetika rasta ovisila je o tijeku fermentacije, a i o upotrijebljenim sojevima kvasaca. K. apiculata je soj s najvećom brzinom specifičnog rasta, postižući maksimalnu gustoću od približno 10^8 stanica/mL. Nakon što su dostigli maksimalnu gustoću, sojevi ne-Saccharomyces brzo su odumirali. Faza opadanja K. apiculata započinje kad Sacch. cerevisiae postane dominantni kvasac, prije nego što etanol i temperatura dostignu vrijednosti pri kojima je inhibiran rast K. apiculata. Sacch. cerevisiae samo se malo razlikovao po svojim značajkama, a maksimalna brzina specifičnog rasta bila je skoro jednaka kao u K. apiculata. Izgleda da brzina proizvodnje etanola tijekom burne fermentacijske faze ovisi o dosegu gustoće rasta ne-Saccharomyces kvasaca.