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Evolution of Yeasts and Lactic Acid Bacteria During Fermentation and Storage of Bordeaux Wines

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The levels of yeasts and lactic acid bacteria that naturally developed during the vinification of two red and two white Bordeaux wines were quantitatively examined. Yeasts of the genera *Rhodotorula*, *Pichia*, *Candida*, and *Metschnikowia* occurred at low levels in freshly extracted grape musts but died off as soon as fermentation commenced. *Kloeckera apiculata* (*Hanseniaspora uvarum*), *Torulopsis stellata*, and *Saccharomyces cerevisiae*, the dominant yeasts in musts, proliferated to conduct alcoholic fermentation. *K. apiculata* and eventually *T. stellata* died off as fermentation progressed, leaving *S. cerevisiae* as the dominant yeast until the termination of fermentation by the addition of sulfur dioxide. At least two different strains of *S. cerevisiae* were involved in the fermentation of one of the red wines. Low levels of lactic acid bacteria (*Pediococcus cerevisiae*, *Leuconostoc mesenteroides*, and *Lactobacillus* spp.) were present in grape musts but died off during alcoholic fermentation. The malolactic fermentation developed in both red wines soon after alcoholic fermentation and correlated with the vigorous growth of at least three different strains of *Leuconostoc oenos*.

The fermentation of grape juice into wine is a complex microbial reaction traditionally involving the sequential development of various species of yeasts, lactic acid bacteria (1, 2, 12, 17), and, perhaps, bacteriophages (24). Yeasts are primarily responsible for the alcoholic fermentation of the juice, which may then undergo another fermentation, malolactic fermentation, by lactic acid bacteria. Traditionally, wines have been produced by natural fermentations caused by the development of yeasts and lactic acid bacteria originating from the grapes and winery equipment. Natural fermentations continue to be the main style of fermentation conducted in France (2, 21), although active dry yeasts are sometimes used in specific conditions when they are able to improve the fermentation process (15). In other countries, such as the United States, Australia, and South Africa, most fermentations are induced by inoculation with selected yeast strains and, in some cases, with selected lactic acid bacteria (2, 12). Nevertheless, any inoculated organisms will always be in addition to those naturally present; the evolution of natural flora is still expected to maintain an influence on the fermentations that develop. Moreover, there is at present uncertainty over whether any inoculated species will develop in dominance to the natural flora (3).

Wine quality is closely related to the microbial ecology of fermentation. The various microbial species that develop during fermentation and the extent to which these individual species grow determine the types and concentrations of the many substances that may contribute to the aroma and flavor characteristics of wine (2, 19, 20). Despite this essential contribution of microorganisms, the ecology of wine fermentation remains only partially understood. Numerous studies (reviewed in references 1, 2, 10–12, 17, and 21) throughout the wine regions of the world have identified the main species of yeasts and lactic acid bacteria that develop during vinification. However, these studies have been largely qualitative or semiqualitative in contribution and mostly describe the frequencies of occurrence of various species at well-separated stages of vinification (5, 7, 9, 18). Quantitative information describing the development of individual species during vinification is lacking. In this study, we examined the quantitative development of individual species of yeasts and lactic acid bacteria during vinification of four quality Bordeaux wines.

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MATERIALS AND METHODS

Wines. The four wines studied were produced under commercial conditions during the 1982 Bordeaux vintage. Red wine A was produced from Merlot variety grapes at a "premier grand cru classe" chateau in the Graves appellation. Red wine B was produced from Merlot variety grapes at a chateau in the Premier Côtes de Bordeaux appellation. The musts of both wines (pH 3.2 to 3.3) were sulfured to give a final concentration of 25 to 30 mg of SO_2 per liter. The yeast flora of the musts was not altered either qualitatively or quantitatively by the SO_2 addition. The sulfured musts were transferred to large metal fermentors (20,000-liter capacity) in which fermentation was conducted at 25 to 30°C. At the end of fermentation, the wine was run off lees into another metal tank for wine A and into oak barrels for wine B. The cellars were heated to 20 to 25°C to induce malolactic fermentation. When malolactic fermentation was largely completed, wine was transferred to a large vessel for mixing, for completion of malolactic fermentation, and for the addition of 25 to 30 mg of SO_2 per liter. Wine A was then transferred to barrels for cellar storage, but wine B was stored in the large mixing vessel. Wine temperature decreased to ca. 6°C during the winter months. Wine A had a final pH of 3.6 and a final ethanol concentration of 12.5%. Wine B had a final pH of 3.5 and a final ethanol concentration of 12%.

White wine C was produced from Semillon grapes at a "premier grand cru classe" chateau in the Sauternes appellation. The grapes were harvested late, after infestation by the mold *Botrytis cinerea*. The must (pH 3.5 to 3.6) was sulfured to 50 mg of SO_2 per liter, clarified by natural sedimentation overnight, and transferred to oak barrels (225 liters) in which fermentation was conducted at 20 to 25°C. Fermentation stopped spontaneously when the residual sugar concentration was ca. 100 g/liter and the ethanol concentra-

tion was 13.5%. At this time, wine from the various barrels was mixed, 50 mg of SO_2 per liter was added, and the temperature was lowered to 5°C to sediment the yeasts. The wine was then returned to oak barrels for subsequent storage. The final pH of wine C was 3.6, and the final ethanol concentration was 13.5%. White wine D was produced from grapes at the same chateau as red wine B. The must (50 mg of SO_2 per liter, pH 3.0) from Semillon grapes was clarified by natural sedimentation and transferred to metal barrels (100 liters) for fermentation at 25 to 30°C. At the end of alcoholic fermentation, 50 mg of SO_2 per liter was added to the wine, which was then separated from yeast sediment by being run off into empty metal barrels for storage. The final pH of wine D was 3.3, and the final ethanol concentration was 12%.

All of the wines were produced by natural alcoholic fermentation with no inoculated yeast. Malolactic fermentation in the red wines was also a natural development. Wine samples (500 ml) were taken at frequent intervals during vinification for enumeration, isolation, and identification of yeasts and lactic acid bacteria. Samples were taken from the middle of fermentation vessels or barrels and immediately transported to the laboratory for microbiological examination. The commencement and duration of the malolactic fermentation was determined at the wineries by malic acid measurements.

Enumeration, isolation, and identification of yeasts and lactic acid bacteria. Yeasts were enumerated by spread inoculating 0.1-ml samples of wine (diluted in 0.1% peptone, if necessary) onto plates of malt extract agar (MEA) (E. Merck AG, Darmstadt, Federal Republic of Germany) and grape juice agar (GJA). GJA (pH 3.0) consisted of half-strength commercially bottled grape juice (without preservatives) and 0.1% yeast extract to which agar was added after sterilization. Plates were incubated at 20 to 25°C for 5 days for colony development. The various colony types were counted, and representative colonies were isolated and subcultured onto MEA for subsequent identification. Yeast isolates were identified by the procedures of Lodder (16). Some of the fermentation and assimilation tests were done with API 20C and 20C auxanogramme galleries for yeasts (API System SA, France) as described by Lafon-Lafourcade and Joyeux (14).

Lactic acid bacteria were enumerated by surface spreading 0.1-ml samples of wine on the following media: MRS agar (6) supplemented with 15% tomato juice (pH 5.5); DB agar (250 ml of tomato juice, 5 g of yeast extract, 5 g of peptone, 5 g of L-malic acid, 1.0 ml of Tween 80, 50 mg of $MgSO_4 \cdot 7H_2O$, 20 mg of $MnSO_4 \cdot H_2O$, 15 g of agar per liter of H_2O [pH 4.5]); and NM agar (10 g of glucose per liter, 5 g of yeast extract per liter, 5 g of Casamino Acids per liter, 5 g of DL-malic acid per liter, 15 g of agar per liter [pH 4.5]) (14). Plates were incubated at 20 to 25°C for 7 to 15 days in jars made anaerobic with GasPaks (BBL Microbiology Systems, Cockeysville, Md.). The various colonies that formed were counted and considered to be lactic acid bacteria on the basis of gram-positive and catalase-negative reactions. Representative colonies were isolated and identified with the API 50CH carbohydrate galleries for lactobacilli as described by Lafon-Lafourcade and Joyeux (14).

RESULTS

Evolution of yeasts and lactic acid bacteria during vinification of red wines. Figures 1a and b show the growth of yeasts and lactic acid bacteria during the vinification of red wines A and B. *Kloeckera apiculata*, *Torulopsis stellata*, and *Saccharomyces cerevisiae* were the dominant yeasts in the freshly extracted musts for both wines. Lower levels (10^3 to 10^4 cells per ml) of *Pichia terricola*, *Pichia kudriavzevii*, and an unidentified *Rhodotorula* species were also present in the must for wine A, and *Pichia kluyveri*, *Saccharomyces fermentati*, and *Rhodotorula glutinis* were also present in the must for wine B. The behavior of yeasts during the fermentation of both red wines was represented by the following events: (i) a rapid decrease and disappearance of the *Pichia* and *Rhodotorula* species (and also *S. fermentati* for wine B); (ii) an initial proliferation of *K. apiculata* to 10^7 to 10^8 cells per ml, followed by a rapid decline and disappearance of this species (Fig. 1a and b); (iii) a rapid growth of *S. cerevisiae* to 10^8 to 10^9 cells per ml to dominate the alcoholic fermentation and the continued survival of the yeasts during the subsequent malolactic fermentation; (iv) a slow initial proliferation of *T. stellata* to 10^6 to 10^7 cells per ml and a gradual decline in these cell levels; and (v) the appearance of *Pichia membranaefaciens* after commencement of the malolactic fermentation and its continued survival in wine at 10^4 cells per

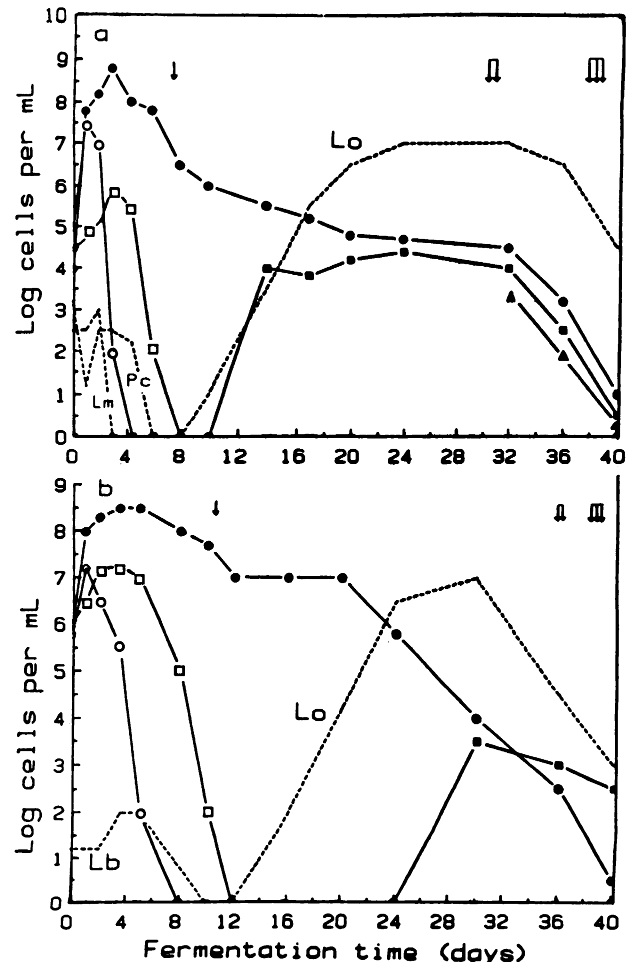

FIG. 1. Evolution of yeasts and lactic acid bacteria during vinification of red wine A (a) and red wine B (b). Symbols: (●) *S. cerevisiae*; (○) *K. apiculata*; (□) *T. stellata*; (■) *P. membranaefaciens*; (▲) *S. bayanus*; (----) growth of lactic acid bacteria *L. mesenteroides* (Lm), *P. cerevisiae* (Pc), *L. oenos* (Lo), and *Lactobacillus* spp. (Lb); (↓) finish of alcoholic fermentation and transfer of wine from fermentor to barrels; (↓↓) remixing of barreled wines in fermentor; (↓↓↓) return of wine to barrels and addition of 30 to 50 mg of SO_2 per liter.

ml. For wine A, *Pichia fermentans* occurred in equal numbers with *P. membranaefaciens*, and *Saccharomyces bayanus* was also isolated (Fig. 1a).

Two different strains of *S. cerevisiae* were responsible for the fermentation of wine B. This was not evident at first, since both strains gave similar colony morphologies on MEA and GJA enumeration plates after standard incubation. However, after storage of these isolation plates at 5°C for 7 days, the colonies of one of the strains became duller and slightly brown in appearance, and on this basis, the two strains could be differentiated. Upon further examination, the two strains exhibited similar sugar fermentation and carbon assimilation reactions but were markedly different in sporulation characteristics. The dull-colony strain produced mostly two spores per ascus, whereas the other strain produced mostly three to four spores per ascus. The dull-colony strain was not present in the early phases of fermentation and was first noticed 3 days after fermentation had commenced.

Samples of both wines were examined at 1- and 2-month intervals after the addition of SO_2 (Fig. 1a and b) and had yeast counts of 10^2 to 10^3 cells per ml. *S. cerevisiae* was not present in these samples, which contained mainly *P. membranaefaciens*, *Candida krusei*, and lower numbers of the haploid species *Saccharomyces bailii* and *Saccharomyces exiguus*.

MEA was a better medium than GJA was for the isolation and enumeration of wine yeasts. Yeast colonies were larger on GJA but were often 5 to 10 times lower in number. This was especially true for *K. apiculata*. *Rhodotorula* species never grew on GJA, and *T. stellata* was sometimes absent on this medium but present on MEA.

With respect to lactic acid bacteria, *Pediococcus cerevisiae* and *Leuconostoc mesenteroides* were each present at 10^2 to 10^3 cells per ml in the must for wine A, but they did not grow and died off by the end of alcoholic fermentation. The presence of *Leuconostoc oenos* was first noticed just after alcoholic fermentation had finished. It grew to a level of 10^7 cells per ml and conducted the malolactic fermentation (Fig. 1a). The viability of *L. oenos* was reduced by the addition of SO_2 at 38 days, but it was still present at 10^3 cells per ml in samples taken 1 and 2 months later. These last two samples also contained 10^4 cells of an unidentified *Lactobacillus* species per ml.

The must for wine B contained very low levels of an unidentified *Lactobacillus* species that died off by the end of alcoholic fermentation. *L. oenos* was subsequently detected, and it conducted malolactic fermentation during its growth to 10^6 to 10^7 cells per ml (Fig. 1b). It remained the only species in wine B and was present at 10^4 cells per ml in samples taken 1 and 2 months after the addition of SO_2.

As indicated above, *L. oenos* was the only species of lactic acid bacteria isolated during malolactic fermentation in both wines A and B. Its development occurred naturally, but this was encouraged by raising the temperature of the wines to 20 to 25°C. Considerable heterogeneity was noted between the various strains of *L. oenos* that were isolated during the course of malolactic fermentation. This strain heterogeneity occurred in both wines and was first recognized as differences between the sizes of colonies that developed on agar enumeration plates. Initially, two types were observed, namely, very small, transparent, "pinhead"-sized colonies and larger, translucent colonies with 1-mm diameters. Both colony types occurred in approximately equal numbers throughout malolactic fermentation, but towards the end of this fermentation, a small proportion (10%) of a third, slightly larger-sized colony had also developed. Differences between colony size were more readily seen on MRS agar plates than on DB or NM agar. Colonies of *L. oenos* developed more slowly on MRS agar than on the other two media, but their counts were ca. 10 times higher on MRS agar. The three colony types retained their characteristic sizes on subculture, but despite their morphological differences, all were identified as *L. oenos* by standard biochemical tests. A total of 30 strains of *L. oenos* were isolated from samples of the two wines taken at various times during malolactic fermentation. All of the isolates produced branched chains of coccobacilli that are characteristic of *L. oenos*, but considerable variation was noted between isolates in the length of the chains, which ranged from 5 to 30 cells. Individual isolates varied in the ability or rapidity with which they fermented ribose, L-arabinose, glucose, or fructose, but these different properties did not correlate with colony size or the stage of isolation during vinification.

Evolution of yeasts and lactic acid bacteria during vinification of white wines. Figures 2a and b show the growth of yeasts and lactic acid bacteria during the production of white wines C and D. *Hanseniaspora uvarum* (wine C), *K. apiculata* (wine D), *T. stellata*, and *S. cerevisiae* predominated in the freshly extracted musts. Slightly lower numbers (10^5 cells per ml) of *Metschnikowia pulcherrima* and *C. krusei* also occurred in the must for wine C, and *Rhodotorula graminis* and *Saccharomyces krusei* (10^4 cells per ml) were also present in the must for wine D. The initial yeast population in wine C must was ca. 10 times higher than that in wine D must, and this is consistent with previous reports on musts from late-picked grapes infected with *Botrytis cinerea* (21).

S. cerevisiae dominated the alcoholic fermentation of both white wines, but *T. stellata* also showed significant growth and survived into the later stages of fermentation (Fig. 2a and b). *H. uvarum*, *M. pulcherrima*, and *C. krusei* exhibited slight growth and survival in wine C but died off by day 7 of the experiment. *K. apiculata* and *R. graminis* rapidly died off without proliferation in wine D, whereas *C. krusei* showed an initial proliferation before its disappearance.

S. cerevisiae was not isolated from either white wine 1 and 2 months after fermentation termination by the addition of SO_2. At these times, *S. bailii*, *Saccharomyces globosus*, *P. membranaefaciens*, and *C. krusei* were isolated from wine C, and *P. membranaefaciens* and *Candida valida* were present in wine D. Counts of these yeasts were ca. 10^3 cells per ml.

Lactic acid bacteria were present at very low levels during the fermentation of both white wines, but they did not multiply and eventually died off. (Fig. 2a and b). *L. mesenteroides* rapidly disappeared from both wines, but *P. cerevisiae* and the unidentified *Lactobacillus* species survived into the fermentation and, presumably, were more resistant to alcohol. No lactic acid bacteria were found in samples of the wines taken 1 and 2 months after the addition of SO_2.

DISCUSSION

The quantitative data presented in this study extend our knowledge of the sequential development of yeast species during natural wine fermentations. Numerous qualitative studies have shown that this sequence is mostly represented by an initial growth and death of apiculate yeasts within the genera *Kloeckera* and *Hanseniaspora*, followed by the growth of *S. cerevisiae*, which then dominates the greater part of alcoholic fermentation. Early proliferation and death of *Hansenula*, *Candida*, and *Torulopsis* species have also

been reported (2, 11, 12, 17). Our data showing the population levels of individual yeast species now provide a more detailed description of the kinetics of yeast behavior during vinification.

The isolation of various *Rhodotorula*, *Pichia*, *Candida*, and *Metschnikowia* species from freshly extracted musts concurs with many other reports (2, 17), although it is noteworthy that we did not find any *Hansenula* species. The rapid death and disappearance of these yeasts from fermenting musts is consistent with their oxidative or weakly fermentative metabolism and sensitivity to ethanol. The slight growth of *C. krusei* and *M. pulcherrima* in white wine musts is noteworthy since these species may lead to increased levels of acetic acid, esters, and higher alcohols in wine (22, 25).

The initial proliferation of apiculate yeasts was most apparent in red wines in which the population of *K. apiculata* increased by 1 to 2 log units. The extent of this growth would be expected to affect wine quality since strains of this yeast are known to produce up to 1 to 2 g of acetic acid per liter and to augment the levels of wine esters and glycerol (2, 22, 25). The reduced ability of apiculate yeasts to grow in white wines may be related to the lower pH of these wines. The inability of apiculate yeasts to sustain their presence in fermenting musts has been attributed to their sensitivity to ethanol concentrations above 5 to 6% (1, 2, 17).

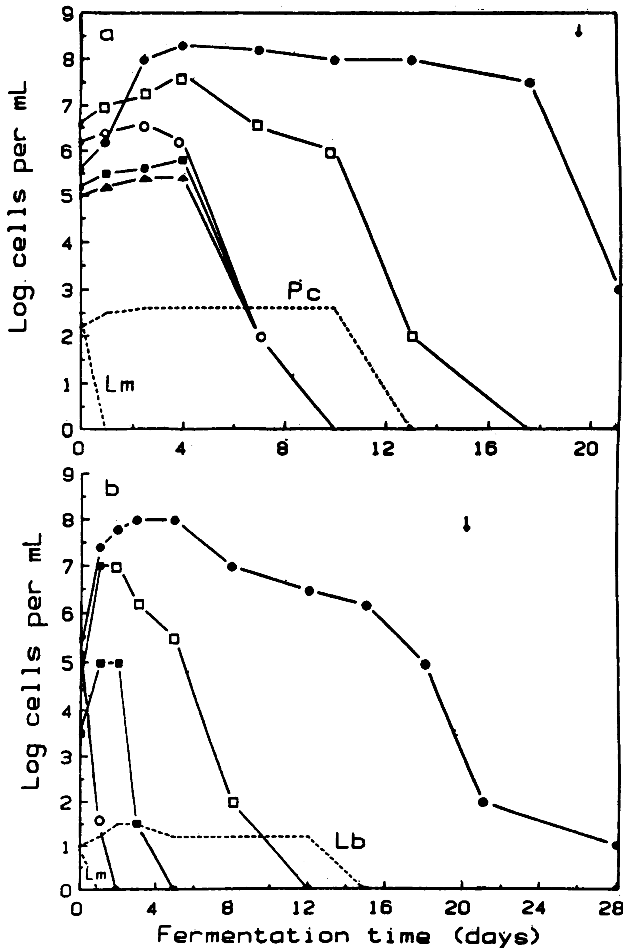

FIG. 2. Evolution of yeasts and lactic acid bacteria during vinification of white wine A (a) and white wine B (b). Symbols: (●) *S. cerevisiae*; (○) *K. apiculata* (*H. uvarum*); (□) *T. stellata*; (■) *C. krusei*; (▲) *M. pulcherrima*; (----) growth of lactic acid bacteria *L. mesenteroides* (Lm), *P. cerevisiae* (Pc), and *Lactobacillus* spp. (Lb); (↓) finish of alcoholic fermentation and addition of 30 to 50 mg of SO_2 per liter.

Next to *S. cerevisiae*, *T. stellata* was quantitatively the most important yeast species recovered from all four wines. The ability of *T. stellata* to grow slowly in fermenting musts to ca. 10^7 cells per ml and its sustained presence almost until the end of alcoholic fermentation suggest that this yeast makes a greater contribution to the ecology of fermentation than previously thought (2). The detection and isolation of *T. stellata* from fermenting musts require special attention and may account for its sporadic isolation in the past. This species grows much more slowly on isolation media than other wine yeasts and produces small colonies that are easily overlooked. The capacity of *T. stellata* to tolerate 10 to 12% ethanol (2, 17; A. Bertrand and E. Soufleros, Ph.D. theses, Université de Bordeaux II, Talence, France, 1975 and 1978, respectively) would account for its continued presence in fermenting musts, and its ability to produce high levels of acetic acid (2, 23), ethyl lactate, and methyl-2-propanol (Soufleros, Ph.D. thesis) may influence wine quality. All of our isolates fermented glucose, sucrose, and raffinose and, as described by Lodder (16), were synonymous with *Saccharomyces rosei*, another well-known wine yeast (2).

In accordance with many earlier studies (2, 5, 7, 9, 18), *S. cerevisiae* grew rapidly in must and dominated alcoholic fermentation. Many different strains of *S. cerevisiae* have been isolated from wine fermentations, and it is supposed that wine character may be influenced by the particular strain conducting the fermentation. This is because different strains produce different concentrations of flavor and aroma constituents, such as acetic acid, esters, higher alcohols, and volatile sulphur compounds (8, 19, 20, 22, 23, 25, 26). The discovery that two strains of *S. cerevisiae* were associated with the fermentation of wine B suggests that some fermentations may be conducted by a mixture of *S. cerevisiae* strains. This raises the novel and interesting possibility of sequential growth of two different *S. cerevisiae* strains during one fermentation. Further exploration of this concept is warranted and would require the development and application of specialized techniques to differentiate strains. Electrophoresis (3, 4) and gas-liquid chromatographic analysis of fermentation products (Bertrand, Ph.D. thesis) may be useful techniques for testing this hypothesis. Conventional isolation and identification techniques do not permit easy distinction of yeast strains.

The growth of *P. membranaefaciens* to levels of ca. 10^4 cells per ml in both red wines during the course of malolactic fermentation was an unexpected observation. This yeast is normally regarded as a spoilage organism that can produce increased levels of acids and esters in wines (2, 22, 25). The development of this species is generally associated with exposure of wine to air, but we consistently isolated it from wine samples taken from the center of barrels during a period of considerable carbon dioxide generation by malolactic fermentation. Further studies are necessary to clarify the presence and significance of this yeast during vinification.

The enological significance of lactic acid bacteria has been well reviewed (10, 12, 13). The occurrence of various *Pediococcus*, *Lactobacillus*, and *Leuconostoc* species in freshly crushed musts and their subsequent death during alcoholic fermentation have been reported previously (6, 13, 21) and were confirmed by our studies. The vigorous growth of *L.*

oenos and concomitant malolactic fermentation soon after the completion of alcoholic fermentation are very characteristic of Bordeaux red wines (21). *Pediococcus* species were not associated with malolactic fermentation, as has been found in some Australian red wines (6). A novel finding of our study was the association of three different strains of *L. oenos* with malolactic fermentation in both red wines examined. We also made the same observation with another Bordeaux red wine but have not presented the data in this report. Strain variation among *L. oenos* isolates has been noted elsewhere (13, 21), but here we report its occurrence in one fermentation. The enological significance of strain variation within *L. oenos* and the possibility that there may be a successive evolution of these strains during malolactic fermentation require further study. In conclusion, our study has further highlighted the complex ecology of yeasts and lactic acid bacteria associated with winemaking. Research and development of methods that differentiate strains of *S. cerevisiae* and that would make possible the selective isolation and enumeration of various species of wine yeasts would contribute to a further understanding of this ecology.

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