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Lactic Acid Bacteria Native to Washington State Wines

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LACTIC ACID BACTERIA NATIVE TO WASHINGTON STATE WINES

Introduction

Malolactic fermentation (MLF) involves the conversion of malic acid to lactic acid with the loss of carbon dioxide by certain lactic acid bacteria (11). Most wineries inoculate with strains of *Leuconostoc oenos,* although other genera of lactic acid bacteria including *Pediococcus* and *Lactobacillus* spp. can also catalyze the fermentation. Lactic acid bacteria have been isolated from wines around the world (37) with several strains of *L. oenos* commercially available to wineries as freeze-dried or liquid cultures.

Little is known concerning the lactic acid bacteria present in wines from Washington State. Since wines from this region commonly have pH values in excess of 3.5 (5, 21, 28), there is increased potential for growth, especially of *Pediococcus* or *Lactobacillus.* This publication details research that isolated and characterized native strains of lactic acid bacteria from commercial wines produced in Washington State.

Isolation and Identification

In 1989, Washington wineries were asked to complete a written survey of enological practices performed at their locations. Using this information, wines were collected from those wineries where MLF was encouraged without using commercially available cultures. This minimized the possibility of reisolating commercial strains. Once isolated in the laboratory, native strains were assigned 1) the prefix "WS" since the strains were isolated from Washington State wines, 2) a number representing the wine lot from which the strain was isolated, and 3) an optional letter indicating that two or more strains were isolated from the same wine sample (lot).

The procedures used to isolate and identify the strains as to genus and species were those detailed by Edwards et al. (12, 14), Edwards and Jensen (13), and Jensen and Edwards (20). Tolerances of native strains to several adverse conditions in wine (low pH, low temperature, ethanol, and sulfur dioxide) were performed using methods previously described in other work(12, 13, 14).

Sources and Distribution

A total of 32 wines were obtained from the 14 wineries that participated in this study. Most of the collected wines were from either tanks or barrels after alcoholic fermentation was completed. Cabernet Sauvignon (10 lots) and Merlot (6 lots) comprised half of the accumulated wines (Figure 1). While most samples were obtained from tanks or barrels after alcoholic fermentation, one Cabernet Sauvignon sample was a press fraction produced from commercial vineyard grapes (Vineyard A). Other wines collected and analyzed were Chardonnay, Sémillon, Chenin Blanc, Sauvignon Blanc, Grenache, Riesling, Pinot Noir, and Royalty.

The sources of the wines along with strain numbers and identification of the bacteria are listed in Table 1. From these wines, a total of 45 strains were isolated and identified. Due to the larger number of lots collected (Figure 1), the majority of the strains (>60%) came from Cabernet Sauvignon, Merlot, and Chardonnay wines. In all, 16 strains of *Leuconostoc oenos*, 10 strains of *Pediococcus* spp. (*P. parvulus* and *P. inopinatus*), and 15 strains of *Lactobacillus* spp. (*L. plantarum, L. brevis, L. hilgardii,* and *L. fructivorans*) were identified from these wines. Four strains (WS-13A, WS-14A, WS-26B, and WS-28A) are believed to be strains of *L. oenos* but the identity could not be confirmed. Similarly, additional strains of *Lactobacillus* were isolated but not identified as to species.

The ecology of lactic acid bacteria during vinification is complex. Different species

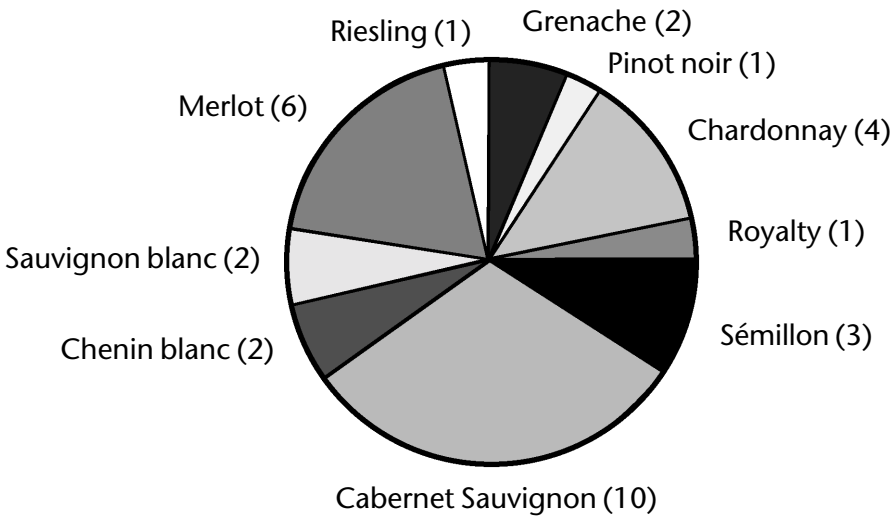

Figure 1. Commer cial wine lots by grape cultivar . Numbers in parentheses refer to the total number of samples collected.

dominate the microflora at different times. As evidence, several wines were found to contain more than one strain or genus of lactic acid bacteria. This observation was best exemplified by wineries C and E where strains of *L. oenos, Pediococcus* spp., and *Lactobacillus* spp. were isolated. Single wine lots were also observed to have more than one bacteria type. This was the case for the one Chardonnay wine obtained from winery C from which WS-4A (*P. parvulus*) and WS-4B (*L. oenos*) were isolated. In a comprehensive study by Costello et al. (6), the authors isolated *Lactobacillus jensenii, L. buchneri, L. hilgardii, L. brevis, L. cellobiosis, L. plantarum, Leuconostoc oenos* and *Pediococcus* spp. from musts and wines at different times during vinification, in agreement with others (9, 10, 16, 24, 33). However, each wine lot obtained for microbiological analysis in this study was only sampled one time during vinification. Thus, it is probable that sampling the same lots at different times would have yielded different species of lactic acid bacteria than those isolated.

Leuconostoc oenos

A total of 16 strains of *L. oenos* were isolated from the commercial wines (Table 1) including Cabernet Sauvignon (3 strains), Merlot (3 strains), Chardonnay (3 strains), Sémillon (2 strains), Pinot Noir (2 strains), Grenache (2 strains), Chenin Blanc (1 strain), and Sauvignon Blanc (1 strain).

The strains of *L. oenos* were characterized to determine tolerances to pH, sulfur dioxide and temperature (12) since these factors can be used by winemakers to select strains. All tests were performed using a synthetic medium rather than wine to limit other adverse conditions (e.g., ethanol) to bacterial growth. The tests compared the growth of native strains with that of the commercially available strains ML-34 (36) and PSU-1 (1).

Generally, most strains grew well at pH 3.3 to 4.5 while none grew at pH 2.9. Some strains, most notably WS-18, WS-21A, WS-27, WS-30, ML-34 and PSU-1 grew better at pH 3.3 than other strains tested. Using media at pH 3.5, strains WS-28B and ML-34 grew the fastest in 30 mg/L total SO_2 while strains WS-17, WS-18, WS-19A, WS-21A, and WS-22 were slower. No growth was observed for PSU-1 at this concentration of SO_2. Finally, strains WS-18 and ML-34 grew best at a lower temperature (12˚C/54˚F) while growth of WS-28B was very slow at this temperature.

Table 1. Sour ces and identification of malolactic bacteria isolated from commercial Washington State wines.

Winery (Vineyard) /wine	Strain	Identification
Vineyard A		
Cab. Sauvignon	WS-2	*L. brevis*
Winery C		
Chardonnay	WS-3A	*L. hilgardii*
	WS-3B	*L. hilgardii*
Chardonnay	WS-4A	*P. parvulus*
	WS-4B	*L. oenos*
Sémillon	WS-5	*Lactobacillus* spp.
Sémillon	WS-6A	*L. oenos*
	WS-6B	*Lactobacillus* spp.
Chenin Blanc	WS-7A	*L. hilgardii*
	WS-7B	*L. oenos*
	WS-7C	*P. parvulus*
Winery D		
Cab. Sauvignon	WS-8	*L. oenos*
Cab. Sauvignon	WS-9	*P. parvulus*
Merlot	WS-10A	*P. parvulus*
	WS-10B	*P. inopinatus*
	WS-10C	*L. oenos*
Cab. Sauvignon	WS-11	*P. parvulus*
Cab. Sauvignon	WS-12	*P. parvulus*
Winery E		
Cab. Sauvignon	WS-13A	(unidentified)
	WS-13B	*P. parvulus*
Merlot	WS-14A	(unidentified)
	WS-14B	*P. parvulus*
	WS-14C	*L. fructivorans*
Winery F		
Merlot	WS-16	*L. plantarum*
Chardonnay	WS-17	*L. oenos*
Winery G		
Cab. Sauvignon	WS-18	*L. oenos*
Pinot Noir	WS-19A	*L. oenos*
	WS-19B	*L. brevis*
	WS-19C	*L. plantarum*
Grenache	WS-30	*L. oenos*
Grenache	WS-31	*L. oenos*
Winery H		
Sauvignon Blanc	WS-20	*L. oenos*
Winery I		
Chardonnay	WS-21A	*L. oenos*
	WS-21B	*L. brevis*
Winery J		
Cab. Sauvignon	WS-22	*L. oenos*
Winery K		
Cab. Sauvignon	WS-23	*L. plantarum*
Merlot	WS-24	*L. brevis*
Merlot	WS-25	*L. oenos*
Chenin Blanc	WS-26A	*Lactobacillus* spp.
	WS-26B	(unidentified)
Sémillon	WS-27	*L. oenos*
Winery L		
Merlot	WS-28A	(unidentified)
	WS-28B	*L. oenos*
Winery M		
Royalty	WS-29A	*P. parvulus*
	WS-29B	*L. hilgardii*

It has been suggested that indigenous bacteria may grow better and induce a faster MLF in wines of that particular region than strains isolated elsewhere (1). Observations of other researchers have supported this hypothesis (2, 7, 35). Thus, the ability of the native strains to induce MLF in Washington wines was evaluated over the course of two years using grapes harvested from experimental plots at the Irrigated Agriculture Research and Extension Center. After completion of alcoholic fermentation and subsequent rackings, the wines was divided into 750 mL lots and inoculated in triplicate with rehydrated lyophilized bacterial cultures at *ca* 10^6 CFU/mL. All wines were kept at 25˚C and the progress of MLF was determined using paper chromatography (22). Methods for must and wine analysis were performed employing standard techniques after completion of MLF (29).

All but 2 strains were able to induce MLF in a 1989 Merlot wine (Figure 2). Most strains completed MLF within 66 days; the exceptions being WS-17 and WS-19A where malolactic activity was not detected 75 days after inoculation. Strains WS-6A and WS-22 completed the fermentation the fastest, requiring only 20 days. Commercially available strains MCW and ML-34 completed the fermentation in 29 and 49 days, respectively. All strains completed MLF in a 1990 Cabernet Sauvignon wine within 44 days while only 50% of the strains completed the fermentation in a 1990 Chardonnay. Overall, strain WS-22 had the best performance in completing the fermentation in the three wines tested.

As expected, wines had higher pH, lower titratable acidities, and higher volatile acidities (VA) upon completion of MLF (Table 2). Normally, VA will increase approximately 0.01 g/100 mL during MLF depending on the strain inoculated (7, 23, 32). Wines inoculated with WS-6A contained the highest amount of VA (0.044 g/100 mL) with the other wines below this concentration. However, all were within generally acceptable concentrations.

Pediococcus spp.

Growth of *Pediococcus* spp. in wines has been considered undesirable due to formation of adverse odors or flavors which reduce quality. As an example, some strains of *P. damnosus* can produce diacetyl and acetoin, odorous compounds often described as smelling like butter and sauerkraut. Several researchers have reported the isolation of *P. cerevisiae* from wines (6, 16, 24, 25, 26), a species name now considered invalid because it represented at least two species including *P. damnosus* and *P. pentosaceus* (17, 31).

Ten strains of *Pediococcus* spp. were isolated from the commercial wines (Table 1) including Cabernet Sauvignon (4 strains), Merlot (3 strains), Chardonnay (1 strain), Chenin Blanc (1 strain), and Royalty (1 strain). Two distinct strains, *P. parvulus* WS-10A and *P. inopinatus* WS-10B, were isolated from one Merlot lot obtained from winery D.

Little is known concerning the ecology and influence of *P. parvulus* on wine quality during vinification. Two of the few studies available describing isolation of *P. parvulus* from wines were those of Davis et al. (9, 10) analyzing Shiraz wines from Australia. While growth of *Pediococcus* spp. in wine depends on inhibitory factors such as SO_2, ethanol, and pH, pediococci can evolve during the course of vinification even after malolactic fermentation catalyzed by *L. oenos.* In fact, *P. parvulus* WS-4A, WS-7C, and WS-10A and *P. inopinatus* WS-10B were isolated from Washington State wines from which native strains of *L. oenos* were also isolated (Table 1). However, the growth of *P. parvulus* in red wines can be quite slow (Figure 3) and not all strains can catalyze MLF. In support, strain WS-9 was the only strain able to complete MLF in a 1990 Cabernet Sauvignon wine unlike strains of *L. oenos* inoculated into the same wine (Figure 2). Strain WS-9 completed the fermentation 100 days after inoculation.

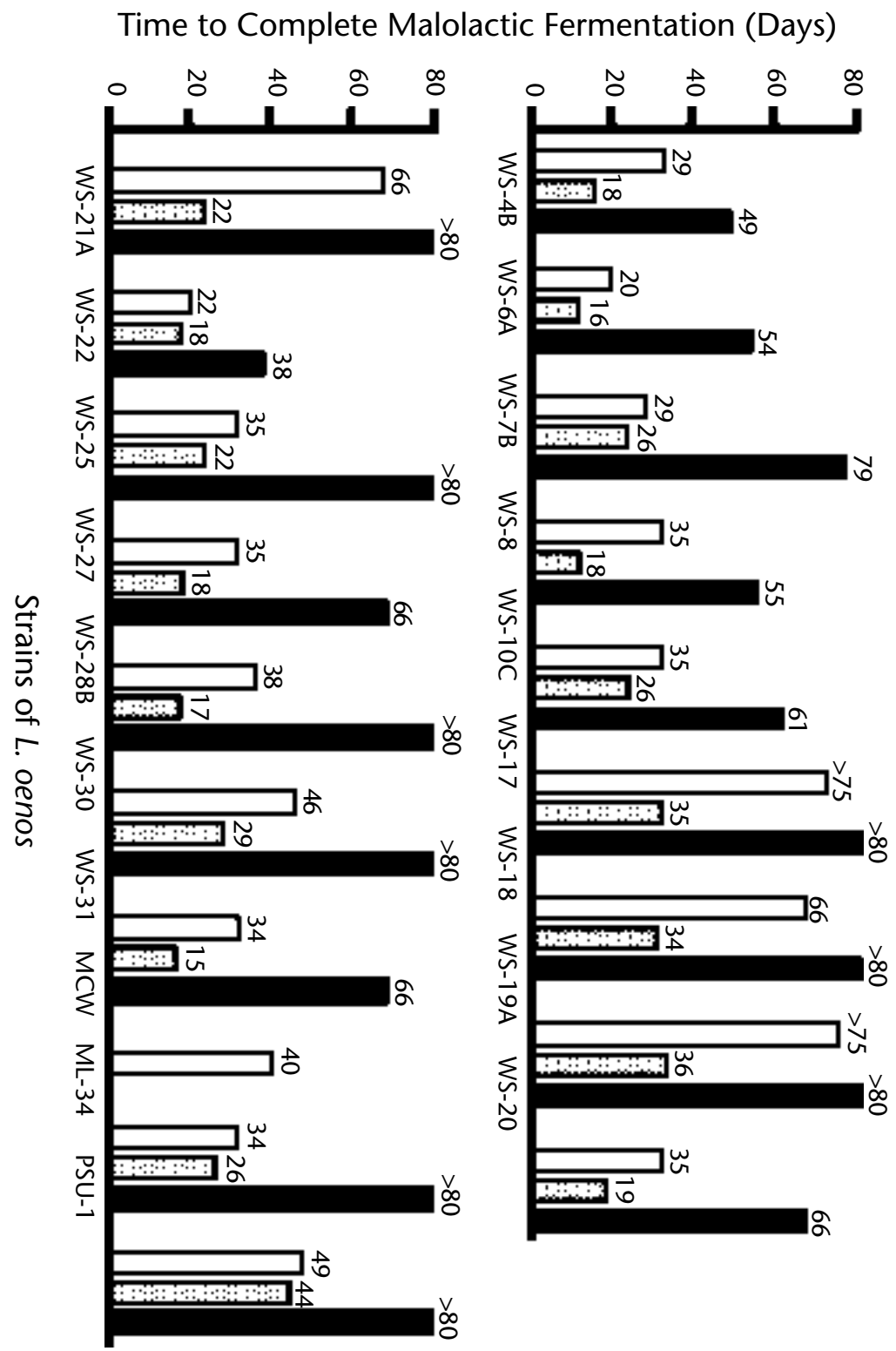

Figure 2. Time to complete malolactic fermentation by different strains of *L. oenos* in a 1989 Merlot (□), a 1990 Cabernet Sauvignon (▒), and a 1990 Chardonnay (■).

Lactobacillus spp.

Uncontrolled growth of *Lactobacillus* spp. in wines can lead to increases in volatile acidity or formation of other adverse odors or flavors. For example, some species/strains can produce diacetyl and acetoin (3, 15). Furthermore, Heresztyn (18) found that *L. brevis* and *L. cellobiosus* produced substituted tetrahydropyridines, compounds thought to be responsible for "mousiness" in wines. However, successful inoculation of a strain of *L. plantarum* into wine to induce malolactic fermentation apparently did not result in an increase in volatile acidity or off-odors (4, 30).

Lactobacillus spp. were distributed in several commercial Washington State wines including Chardonnay (3 strains), Merlot (3 strains), Cabernet Sauvignon (2 strains), Semillon (2 strains), Chenin Blanc (2 strains), Pinot Noir (2 strains), and Royalty (1 strain) (Table 1). Five strains were isolated from winery C. Strain WS-2 was isolated from a press fraction of a fermenting Cabernet Sauvignon must where the grapes were obtained from vineyard A. Species identified were *L. brevis* (4 strains), *L. hilgardii* (4 strains), *L. plantarum* (3 strains), and *L. fructivorans* (1 strain).

It has been generally believed that *Lactobacillus* spp. can not tolerate a pH less than 3.5 (37). If this generalization is correct, wines at pH 3.5 or less should be at less risk of *Lactobacillus* infection than wines of higher pH. However, this does not appear to be the case. As indicated in Table 3, although growth of most strains was slowed in media of pH <3.5, several strains of *L. brevis* and *L. plantarum* could grow well at relatively low pH (pH 3.16 and 3.34). Tolerance to low pH appears to be dependent on the species since *L. hilgardii* or strains WS-5 and WS-6B could not grow at pH 3.16. One consequence of these data is that pH

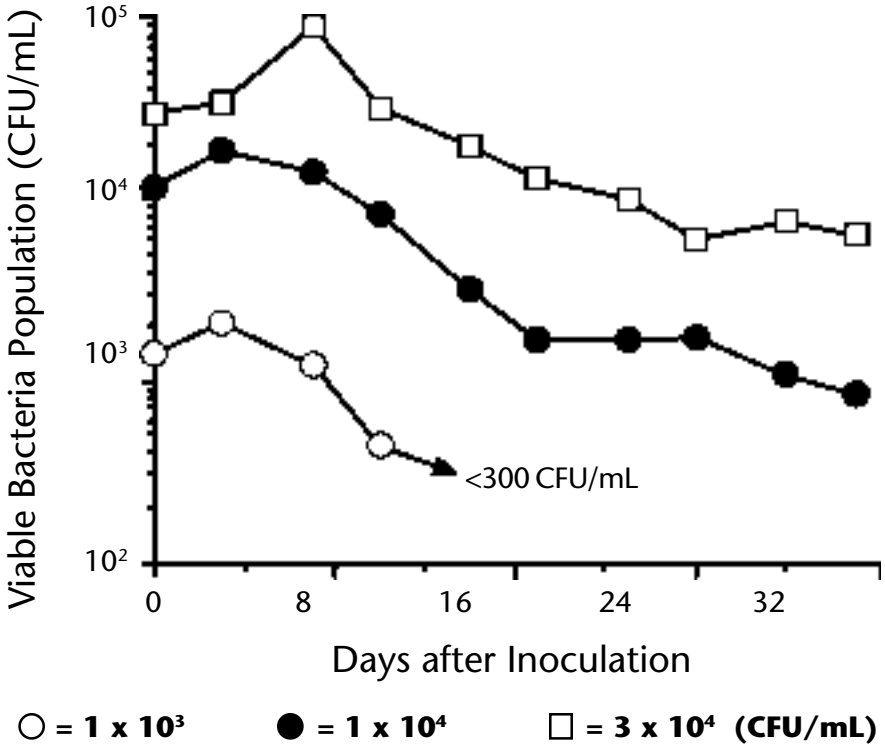

Figure 3. Growth of P. parvulus WS-10A in Cabernet Sauvignon and Merlot wine blend (54%/46%) at different initial bacterial populations.

Table 2. Chemical analysis of 1989 Merlot and 1990 Cabernet Sauvignon wines inoculated with different strains of *Leuconostoc oenos*.

	Merlot			Cabernet Sauvignon		
Strain	pH	TA[1]	VA[2]	pH	TA	VA
None	3.55	0.72	0.018	3.98	0.67	0.035
WS-4B	3.78	0.50	0.037	4.23	0.44	0.053
WS-6A	3.77	0.51	0.044	4.24	0.44	0.054
WS-7B	3.78	0.52	0.039	4.26	0.44	0.057
WS-8	3.75	0.51	0.033	4.23	0.41	0.055
WS-10C	3.75	0.52	0.038	4.26	0.44	0.056
WS-17	–[3]	–	–	4.29	0.42	0.051
WS-18	3.78	0.49	0.030	4.28	0.44	0.057
WS-19A	–[3]	–	–	4.28	0.43	0.053
WS-20	3.79	0.50	0.029	4.27	0.42	0.055
WS-21A	3.77	0.51	0.033	4.25	0.45	0.047
WS-22	3.77	0.52	0.035	4.19	0.44	0.062
WS-25	3.80	0.51	0.034	4.25	0.44	0.045
WS-27	3.81	0.50	0.030	4.24	0.41	0.061
WS-28B	3.78	0.51	0.037	4.23	0.43	0.055
WS-30	3.83	0.51	0.036	4.28	0.45	0.054
WS-31	3.81	0.51	0.032	4.23	0.48	0.045
MCW	3.81	0.51	0.036	–[4]	–	–
ML-34	3.78	0.51	0.035	4.22	0.45	0.054

[1]Titratable acidity (g tartaric acid/100 mL).

[2]Volatile acidity (g acetic acid/100 mL).

[3]MLF not completed >75 days after inoculation.

[4]Not inoculated.

may not control lactic acid bacteria by itself. However, a synergistic effect exists between pH, ethanol level, and cell concentration and the growth of lactic acid bacteria (34) and was not taken into account for these experiments.

Sulfur dioxide has long been used to control the growth of undesirable wine microorganisms, including *Lactobacillus* spp. (8, 11, 37). The extent of bacterial inhibition by sulfur dioxide is largely dependent on the pH of the wine. Low pH favors a higher concentration of undissociated or molecular sulfur dioxide which is the active form of SO_2. The ability of the strains of *Lactobacillus* spp to grow in different concentrations of SO_2 in an MR medium (pH 3.5) is illustrated in Table 3.

In recent years, winemakers have experimented with using little or no SO_2 during grape crush. One consequence of reduced use of SO_2 in wineries is that *Lactobacillus* spp. could theoretically grow and produce excessive volatile acidity or other off-odors or flavors. Although most strains studied grew in 3 to 21 mg/L SO_2 (pH 3.5), The general recommendation is to add a minimum of 30 mg/L SO_2 at grape crush, since growth of all strains was delayed if not inhibited at this concentration (Table 3). Use of SO_2 can be especially important in musts with high populations of *Lactobacillus,* high pH, or if yeast inoculation is delayed a few days.

Another consequence of reduced SO_2 at crush may be a sluggish or stuck alcoholic fermentation. This observation is believed to be due to excessive growth of *Lactobacillus*, although research is not available to support this conclusion. However, some researchers have reported that early inoculation of malolactic bacteria can inhibit alcoholic fermentation (11). In the present study, one strain of concern may be *L. brevis* WS-2. This strain was isolated from a press fraction made from grapes which historically had problems with stuck fermentations.

To study this problem, Concord juice concentrate was reconstituted to 21˚Brix and diammonium phosphate and yeast extract were added as fermentation adjuvants. Strains of *Lactobacillus* spp. were inoculated into the juice at approximately 10^5 CFU/mL. After three days, yeast (*Saccharomyces cerevisiae* Montrachet) was inoculated and the fermentations were monitored gravimetrically (19).

None of the strains tested slowed the alcoholic fermentation in comparison to the control wine without *Lactobacillus* inoculation (Figure 4). Interestingly, the decline in soluble solids was accelerated in the presence of strain WS-21B, unlike the other fermentations, probably due to concurrent growth of *Lactobacillus* and yeast. This experiment indicates that the presence of *Lactobacillus* does not necessarily result in stuck alcoholic fermentations. However, firm conclusions about the ability of other strains or species of *Lactobacillus* to inhibit alcoholic fermentation can not be made since only three species, *L. hilgardii*, *L. brevis,* and *L. plantarum,* were studied. Moreover, the growth of *Lactobacillus* spp. during fermentation was not evaluated. Thus, additional research to evaluate the relationship between the incidence of stuck fermentation and growth of *Lactobacillus* is needed using different species and strains.

Summary and Conclusions

Wibowo et al. (38) stated that it is unrealistic to expect a single strain of *L. oenos* to catalyze MLF under all conditions and in all wines. The current research supports this contention since the strains characterized all possess different tolerances to the adverse conditions found in wines (low temperature, low pH, and/or high SO_2 concentration) and different abilities to catalyze MLF in different wines. Furthermore, the decision of which strain to use cannot be limited to wine conditions in light of observations regarding the impact of strains on sensory quality. In a study by McDaniel et al. (27), strains of L. oenos inoculated into Pinot Noir wines produced in Oregon differentially altered the sensory characteristics of the wine. These data were in agreement with those of

Rodriguez et al. (32) studying Chardonnay wines. Thus, additional research is needed to evaluate the influence of the different strains on the sensory quality of wines. Evaluation of native strains with regard to sensory quality would allow winemakers to impart specific characteristics to wines through selection of bacterial strains, an overall improvement in microbiological control.

The significance of isolating different species of Pediococcus and Lactobacillus from commercial wines and the overall impact on wine quality remains unknown. However, Davis et al. (8) pointed out that it is quite possible that some strains or species of Pediococcus or Lactobacillus may contribute desirable characteristics to wine quality even though excessive growth can be undesirable. This hypothesis is supported by the fact that most of the wines from which these strains were isolated were "not spoiled" in the opinion of the winemaker(s) interviewed for this study. Whether the quality was a result of 1) growth of certain species and strains, 2) metabolic interactions between different species or strains also isolated from these wines, 3) winemaking practices at the winery, or 4) a combination of these factors, remains unknown. Research is continuing to study the ecology of these organisms in wine and their impact on wine quality.

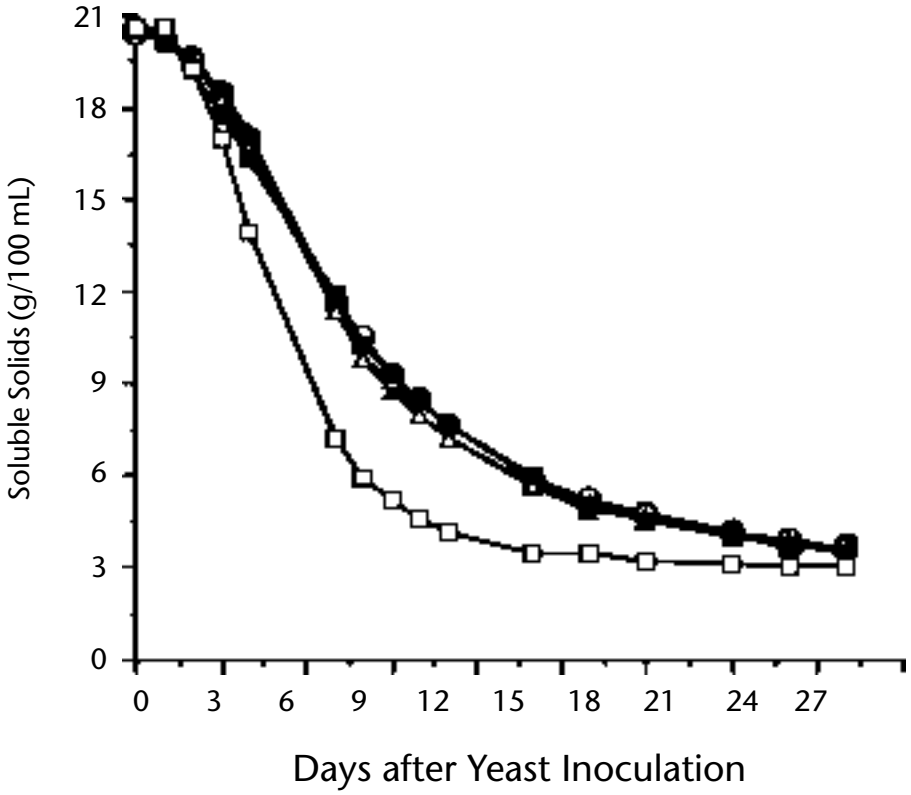

Figure 4. Rate of alcoholic fermentation in reconstituted Concord juice inoculated with yeast (●) or with yeast and *Lactobacillus* spp. strains WS-2 (○), WS-3B (▲), WS-16 (△), WS-21B (□), or WS-23 (■). All yeast inoculations were made 3 days after *Lactobacillus* spp. inoculations.

Table 3. Growth of *Lactobacillus* spp. in MR broth after 6 days at different pH and concentrations of sulfur dioxide (pH 3.56).

	pH				Total SO_2 (mg/L)				
	3.16	3.34	3.59	3.74	0	3	21	33	47
L. brevis									
WS-2	±	++	++	++	++	++	++	–	–
WS-19B	+	++	++	++	++	++	++	–	–
WS-21B	+	++	++	++	++	++	++	–	–
WS-24	±	++	++	++	++	++	++	–	–
L hilgardii									
WS-3A	–	+	++	++	++	++	++	–	–
WS-3B	–	+	++	++	++	++	+	–	–
WS-7A	–	+	++	++	++	++	+	–	–
WS-29B	–	+	++	++	++	++	±	–	–
L. plantarum									
WS-16	+	++	++	++	++	++	++	–	–
WS-19C	±	++	++	++	++	++	++	–	–
WS-23	++	++	++	++	++	++	++	–	–
L. fructivorans									
WS-14C	–	–	+	++	++	+	–	–	–
Lactobacillus spp.									
WS-5	–	±	++	++	++	++	++	–	–
WS-6B	–	±	++	++	++	++	++	+	–

(–) no growth, (±) weak growth, (+) growth, and (++) strong growth

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